CHARACTERIZING MICROALGAE (*Nannochloris oculata*) HARVESTING BY ALUMINUM FLOCCULATION

A Thesis

by

RYAN T. DAVIS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2011

Major Subject: Biological and Agricultural Engineering

Characterizing Microalgae (*Nannochloris oculata*) Harvesting by Aluminum Flocculation

CHARACTERIZING MICROALGAE (*Nannochloris oculata*) HARVESTING BY ALUMINUM FLOCCULATION

A Thesis

by

RYAN T. DAVIS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Co-Chairs of Committee,	Zivko Nikolov
	Ron Lacey
Committee Members,	Timothy Devarenne
Head of Department,	Stephen Searcy

August 2011

Major Subject: Biological and Agricultural Engineering

ABSTRACT

Characterizing Microalgae (*Nannochloris oculata*) Harvesting by Aluminum Flocculation. (August 2011)

Ryan T. Davis, B.S., University of Arizona

Co-Chairs of Committee: Dr. Zivko Nikolov
Dr. Ron Lacey

Recent progress in algae biotechnology indicates that microalgae have the potential of becoming a significant source for food, feed proteins, nutraceuticals, and lipids for biofuels. Typically low concentrations of microalgae cultures (less than 2 g/L) make harvesting of algae biomass one of the key economic bottlenecks for microalgae production of biofuels and bioproducts. Among the various biomass harvesting options currently under consideration, flocculation appears to be the least expensive and most flexible method for harvesting and initial concentration of dilute algal cultures. In addition to initial biomass concentration, processing factors that could also affect harvesting efficiency include culture pH, flocculant dosage, and media ionic strength (conductivity). This thesis reviews challenges of harvesting and concentration of green microalgae and examines the effect of pH, flocculant dosage, and culture conductivity on charge neutralization and flocculation of *Nannochloris oculata* by aluminum chloride.

N. oculata flocculation was studied by manipulating the culture pH and ionic strength before the addition of aluminum chloride. The removal efficiency,

concentration factor, settling rate, and zeta potential of the culture were measured to assess the effect of processing variables and understand mechanisms that govern *N. oculata* flocculation by aluminum chloride. Flocculation tests conducted with culture concentrations of 10^7 cells/ml revealed that $AlCl_3$ concentration of 0.05 g/L and flocculation pH of 5.3 were optimal conditions for achieving 100% removal efficiency and a twentyfold algae concentration. At flocculant concentrations greater than 0.05 g/L, removal efficiencies were equally good but resulting concentration factors decreased with increasing $AlCl_3$ dosage. Zeta potential measurements were correlated with flocculation dosage, initial cell concentration, medium pH, and aluminum solubility curves to conclude that densely charged multi-valent aluminum hydroxide species were responsible for the efficient flocculation at pH 5.3 with 0.05 g/L $AlCl_3$.

DEDICATION

To my Mom

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Nikolov, for his patience and guidance, and my committee members, Dr. Lacey and Dr. Devarenne, for their assistance and mentorship. I would also like to acknowledge and thank Dr. Karthikeyan of Biological and Agricultural Engineering and Dr. Christie M. Sayes and Mike Berg of Veterinary Physiology & Pharmacology, whose generous collaboration and lab use was critical to the completion of this study. I would also like to thank Andrea Garzon, Georgia Barros, and Dr. Lisa Wilken for their direct assistance with the research and development of this study. Thank you as well, to Dr. Wilken for the invaluable editing assistance during the writing process. A special thank you goes to my friends and colleagues, Lou and Jola Brown, for their hard work in Pecos to advance sustainable algae biotechnology.

Thank you to my friends, colleagues, and department faculty and staff for enabling me to succeed. Thank you especially to Sonya Stranges for her invaluable leadership and advice. I also want to extend my gratitude to the National Alliance for Advanced Biofuels and Bio-Products (NAABB), which provided the funding and support for this study. Finally, thank you to my family for their encouragement and support.

NOMENCLATURE

AFDW	Ash Free Dry Weight
Alum	Aluminum Sulfate
CF	Concentration Factor
CFU	Culture Forming Unit
DAF	Dissolved Air Flotation
DHA	Docosahexaenoic Acid
DO	Dissolved Oxygen
DW	Dry Weight
EF	Electrolytic Flocculation
EOM	Extracellular Organic Material
EPA	Eicosapentaenoic Acid
MW	Molecular Weight
NAABB	National Alliance for Advanced Biofuels and Bio-Products
OD	Optical Density
PACl	Polyaluminum Chloride
PBR	Photo Bioreactor
RE	Removal Efficiency
RI	Refractive Index
RPM	Revolutions Per Minute
TSP	Total Soluble Protein
TSS	Total Suspended Solids

UTEX	The Culture Collection of Algae at The University of Texas
W/W	Weight of Solids / Weight of Liquid

TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

1. INTRODUCTION

The Department of Energy has shown that the United States (US) transportation energy requirements can be supplemented with 140 billion gallons of biodiesel per year (Chisti, 2007). Traditional energy crops, such as corn and soy, are not realistic options for providing a significant fraction of this biofuel need because the use of these food crops to fuels creates economic and logistical hurdles and drastically increases the price of food commodities (Chisti, 2007). The only viable option to supplementing the United States' transportation energy requirements with clean renewable fuel is by utilizing algae. Algae have a higher growth rate than plants and can be grown in desert regions where they do not compete with arable land for food crops.

Algae energy content is stored in the form of triglycerides and sugars that can be converted into biodiesel and other types of fuels. Several algae species such as *Neochloris oleoabundans* , *Porphyridium cruentumm, and Botryococcus braunii* can accumulate more than 50% triglycerides on a dry weight basis (Becker, 2008). Combining a relatively fast algae growth rate and a conservative assumption of 30% oil accumulation leads to an estimate of annual algae-oil yield of 58,700 L/ha. This estimate is significantly higher than that of corn and soybeans, which annually yield only 176 and 446 L/ha, respectively.

Besides being able to utilize non-arable land, algae production can reduce CO_2 accumulation in the environment by sequestering large quantities of CO_2 emitted from

---

This thesis follows the style of the Journal of Bioengineering and Biotechnology.

coal-fired power plants, cement factories, and other CO_2-generating sources. Algae are naturally efficient CO_2 scrubbers that sequester CO_2 as a nutrient source, while producing energy rich byproducts such as lipids and carbohydrates (Chisti, 2007). There are still many hurdles to overcome before CO_2 sequestration and biofuel production from algae can become a commercially viable option. To overcome some of these hurdles, scientists and engineers are working on improving photosynthesis efficiency to increase CO_2 uptake, oil accumulation, and growth rate, while finding less expensive ways to harvest and extract algal oil.

There are currently over 100 industrially used species of microalgae for the production of food grade chemicals, nutraceuticals, feed, and biofuels (Becker, 2008). Some of these species, such as *Spirulina sp.* and *Dunaliella sp.*, have been used for protein production because of their high protein content (50-70 % dry weight) (Chisti, 2007). Other species such as *Porphyridium cruentum* and *Neochloris oleoabundans*, which can accumulate oil up to 70 % (w/w) and are more suitable for biofuel production (Chisti, 2007). Hydrocarbons, which can be converted into specialty solvents or gasoline, can be produced by *B. braunii* at up to 90 % (w/w). The genetic manipulation of these organisms could increase the accumulation level of targeted compound and reduce overall production cost. Another way to reduce the cost of production is to increase the density of the algae cultures by increasing the growth rate. Algae are typically grown to a low density of 1 g/L, 0.1 % of total suspended solids (TSS) in outdoor ponds. Inexpensive open ponds do not provide optimal conditions for light utilization and efficient mixing. More expensive closed photo-bioreactors (PBRs)

expose algae to more light and better mixing, which could result in an increase of algae density up to 4 g/L (0.4 % TSS) (Chisti, 2007).

Irrespective of bioreactor configuration, high operating cost of harvesting and concentration of microalgae (flocculant and/or energy cost) could be a substantial development hurdle. The small size of the microalgae (< 20 μm) and low culture densities (0.1-0.4 % TSS) require energy-intensive processes for harvesting and dewatering. For example, harvesting and drying of algae biomass can account for up to 30% of the total biomass production cost and is being cited as a bottleneck preventing scale up and commercialization of algae biotechnology (Gudin, 1986; Uduman et al., 2010). Simple, low-energy mechanical separation techniques currently used for harvesting filamentous algae (>20 μm) are ineffective for harvesting dilute microalgae cultures. Low-cost harvesting techniques such as flocculation and filtration are attractive, but still require subsequent biomass dewatering/concentration and drying before oil extraction. Centrifugation is a commonly used harvesting and concentration processes but is considered too expensive for economical harvesting of dilute microalgae culture when compared to flocculation and filtration. Disc-stack centrifugation can concentrate algal cultures to 3 % TSS in a single pass and to more than 15 % TSS with additional passes through the centrifuge (Molina et al., 2003; Shelef et al., 1984). While high-energy and low throughput disc-stack centrifuges can harvest dilute cultures directly from the pond or photobioreactors, decanter centrifuges require pre-concentrated algal slurry of at least 2 % TSS (Shelef et al., 1984). Centrifugation of pre-

concentrated algal slurries is considered a viable option if the cost of the pre-concentration step is sufficiently low (Schlesinger, 2011; Shelef et al., 1984).

Algae flocculation is considered a superior harvesting method to filtration and centrifugation because it is less sensitive to microalgae size, shape, and specific gravity. Flocculation is best suited as a primary concentration step for processing large volumes of dilute cultures. Achievable concentration factors by flocculation are as high as 200 fold yielding algal slurries of about 1-7% TSS (Uduman et al., 2010). Such pre-concentrated slurries are suitable feed for additional concentration by belt filtration or decanter centrifugation due to their increased particle flock size and specific gravity. Filtration and centrifugation can dewater pre-concentrated algae slurries (2 - 4 % TSS) into an algae paste of 18-25 % TSS.

Flocculation of microalgae can be accomplished using inorganic and polymeric flocculants. Ionic flocculation primarily uses tri-valent aluminum and iron cations. Most of the previous work and know-how for ionic and polymeric flocculants originated from water treatment studies. Objectives and criteria for harvesting microalgae for biofuel and feed production differ from those in water treatment (Uduman et al., 2010). In biofuel production, maximizing biomass recovery and concentration are critical to cost-efficient oil extraction from the dewatered biomass. In addition, the choice of flocculation methods must be compatible with lipid extraction and should not introduce toxic residues that could prohibit water/media recycle or the use of defatted biomass for feed applications.

The efficiency of flocculation processes is related to the processing conditions that may include culture pH and ionic strength, presence of extracellular organic material (EOM), culture age, flocculant concentration, and initial cell density. Ionic flocculation is directly affected by pH, with tri-valent metallic ions working best below pH 6. Polymeric flocculation is relatively unaffected by pH, but its efficiency is inversely related to culture ionic strength (conductivity). Combinations of harvesting and concentration techniques outlined in Figure 1.1 provide some of the cheapest options for dewatering of algae biomass (Becker, 2008). The applicability of suggested processes in biofuel production would depend on algae species, operating conditions, and separations objectives i.e. biomass harvesting vs. treating algae laden waters. In addition, further research is required to determine the effect of selected harvesting and concentration methods on potential biomass contamination with flocculants and filter aid, oil extraction, cell rupturing during centrifugation or electrolytic flocculation, biomass spoilage during drying and storage, and centrifugation efficiency of pre-concentrated algal slurries.

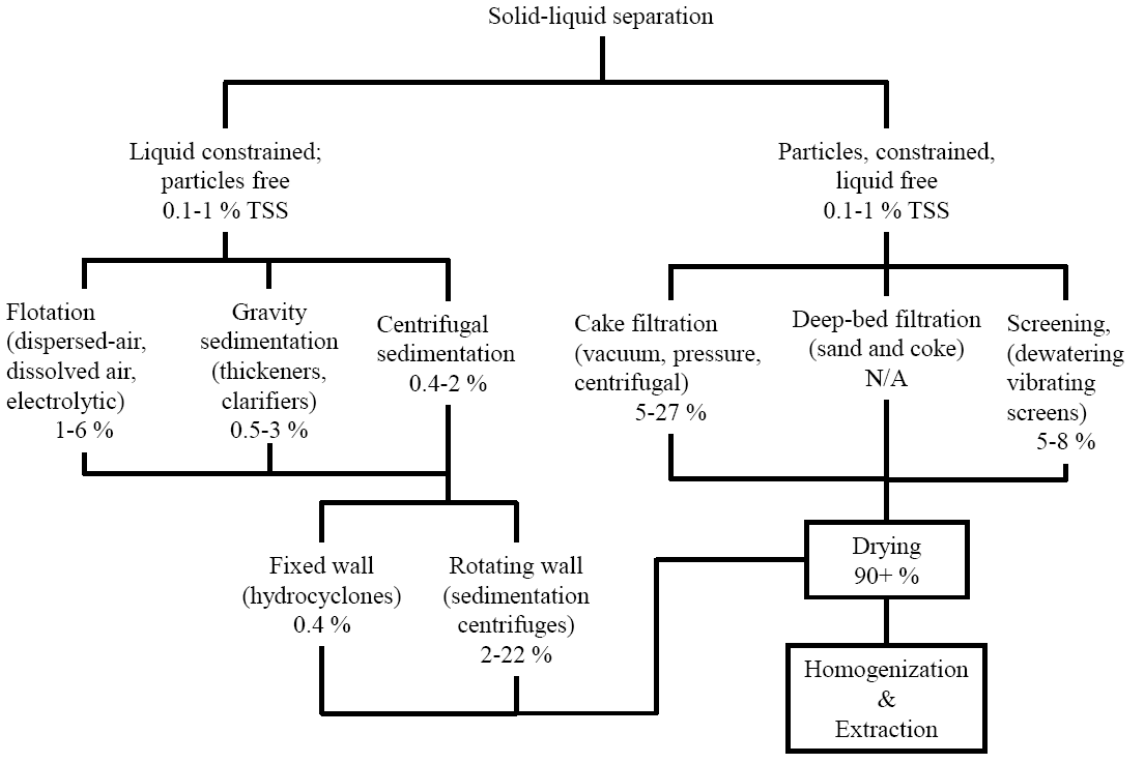


Figure 1.1. Flow diagram of algae solid-liquid separation techniques with final solids % yields (Adapted from (Shelef et al., 1984)).

The goal of this study was to examine the effect of pH, flocculant dosage, and NaCl concentration on the efficiency and mechanisms of $AlCl_3$ flocculation and settling of *Nannochloris oculata*. This thesis examines how these factors affect flocculation removal efficiency, concentration factor, as well as the mechanisms of flocculation including double layer compression, and charge neutralization.

The specific objectives of this project were:

- Characterize optimum flocculation conditions by measuring cell removal efficiency and biomass concentration factor with minimum effective aluminum chloride dosage.
- Determine the effects of pH, flocculant dosage, and NaCl concentration on aluminum chloride flocculation.
- Examine effects of pH and aluminum chloride dosage on charge neutralization by measuring zeta potential.

2. LITERATURE REVIEW

2.1 Microalgae: Definition, Varieties, and Properties

Nannochloris oculata is a small, 1-3 μm diameter spherical unicellular green microalgae species that does not form organized colonies known as filaments. The small size of *N. oculata* creates potential harvesting and processing hurdles that will be the target of this thesis. Species such as *Spirulina* are easier to harvest because of their larger size which can form organized colonies that are similar in structure to filamentous microalgae. Green microalgae are currently targeted as a potential source for next generation biofuels because of their photosynthetic capability, high growth rate, and high nutrient and energy content including lipids, carbohydrates, and protein (Ferell, 2010).

A range of algae species with high nutrient content are detailed in Table 2.1. *Nannochloris oculata* is currently being used in pilot tests because of its heat tolerance, growing in temperatures as high as 38 °C (Brown, 2010). *Nannochloropsis salina* and *Nannochloropsis oculata* are similar to *Nannochloris oculata* and are capable of high growth rates and lipid content that surpass those of commonly grown algae such as *Scenedesmus*, *Chlorella*, and *Dunaliella* species (Gouveia and Oliveira, 2009). *Nannochloris oculata* is currently classified by the following kingdom to strain hierarchy of *Eukatyota, Plantae, Viridaeplantae, Chlorophyta, Chlorophytina, Tetraphtae, Chlorophyceae, Chlorococcales, Coccomyxaceae, Nannochloris, oculata*.

Table 2.1. Gross chemical composition of human food sources and different algae (% of dry matter) (Adapted from (Becker, 2008)).

Commodity	Protein %	Lipid %	Carbohydrate %
Baker's yeast	39	1	38
Corn[a]	4	9	**71**
Egg	**47**	**41**	4
Meat muscle	43	34	1
Botryococcus braunii	-	**90**[b]	-
Spirulina maxima	**60-71**	13-16	6-7
Spirulina platensis	**46-63**	8-14	4-9
Chlorella vulgaris	**51-58**	14-22	12-17
Chlamydomonas rheinhardtii	**48**	21	17
Scenedesmus obliquus	**50-56**	12-14	10-17
Synechococcus sp.	**63**	15	11
Tetraselmis maculate	**52**	15	3
Dunaliella salina	**57**	**32**	6
Dunaliella bioculata	**49**	4	8
Anabaena cylindrical	**43-56**	**25-30**	4-7
Porphyridium cruentum	28-39	**40-57**	9-14
Prymnesium parvum	28-45	**22-38**	25-33
Neochloris oleoabundans[c]	-	**65**	-
Nannochloris oculata[d]	**23**	**30**	-

(a) (Belyea et al., 2004).
(b) *B. braunii* produces long chained hydrocarbons instead of fatty acid lipids.
(c) (Gouveia and Oliveira, 2009).
(d) (Brown, 2010).

N. oculata is a salt water species cultured in the common nutrient buffer Erdschreibers media (Table A.1.2). Algae cells are held in suspension because of the cells low density, which is close to that of water, and negatively charged cell wall. *N.*

oculata does not have a published lipid content, but has been measured at 23% after stressing, has an observed growth rate of 0.15 g/L/d, and a measured cell wall charge of -28 mV (Brown, 2010).

2.1.1 Bioreactors

Algae are grown in specialized bioreactors that can manipulate the algae growth environment by controlling temperature, mixing, nutrients, pH, and light inside the reactor. There are two types of bioreactors; photo-bioreactors and closed bioreactors similar to stainless steel reactors used in microbial fermentations (Becker, 2008; Harrison, 2003). PBRs are used to grow photosynthetic algae for low value protein and oil, while closed bioreactors are used for heterotrophic cultures producing higher value products such as recombinant proteins, hydrogen, and hydrocarbons. Algae can grow as both auto and heterotrophs with photosynthesis assimilating CO_2 in sunlight, while organic carbon is metabolized at night. PBRs are able to efficiently use solar energy by either exposing the algae to the sun light in outdoor ponds, or through clear PBRs made of polyethylene tubing or Plexiglas. The goal of PBRs is to maximize culture growth rate by utilizing as much light as possible with minimal space, cost, and contamination. PBRs can either be open pond types or closed transparent closed reactors. Major designs include circular pond with a rotating agitator, a single or multiple oblong pond with paddle wheel, and sloped ponds with a circulating pump (Becker, 2008).

The Rotating arm design came from water treatment for use as oxidation ponds, while the rest of the designs have more common use in aquaculture. The rotating arm

and paddlewheel are more difficult to scale with issues of inefficient paddle wheel mixing and material cost. Sloped ponds use gravity to propel algae through the raceway with higher efficiency pumps to overcome the resultant head loss. The principle cost of these ponds is the construction and lining cost. The ponds must be lined to prevent silt suspension, percolation, and contamination. The cost of pond lining is \$1-10/m^2 with UV-resistant polyvinyl chloride or white reinforced polyethylene sheets (Becker, 2008). Additionally, concrete is considered too expensive, and requires rebar to stabilize concrete from cracking. Open ponds have been shown to grow microalgae sp. at a rate of 20 g/m^2, with volumetric rates of 0.1 g/L/d (Chisti, 2007). Closed PBRs showed similar per area growth of up to 40 g/m^2, however closed PBRs ability to specifically control the algae's growth environment allow for significantly higher growth rates of 1.5 g/L/d (Chisti, 2007). PBRs can also grow culture densities to 4 g/L while open ponds can only grow to 1 g/L AFDW (Brown, 2010; Chisti, 2007). This difference in density coupled with volume differences per hectare gave a 38% lipid yield per hectare increase in closed PBRs over open ponds (Chisti, 2007).

There are several kinds of PBRs such as Polyethylene sleeves (25 L), Plexiglas tubes, or flat panel reactors. Plexiglas offers an advantage of being able to be hung horizontally or tilted to face the sun, but is more expensive than polyethylene. Despite the higher density and growth rate with closed PBRs, several factors including cost, heat management, and longevity make their use less attractive. Closed PBRs are very expensive to produce compared to open ponds and have pour heat management because they cannot themselves by evaporation like ponds. Closed PBRs must be sprayed with

mist, submerged in a water bath, or shaded which can decrease light utilization. PBRs also have a lower lifetime than open ponds, whose lifetime of 20+ years is attributed to its simplified design.

Closed PBRs have several advantages over open ponds that necessitate their use for large scale algae cultivation. Closed PBRs can grow sterile cultures, while open ponds are readily exposed to contamination, which can decrease productivity. Contamination in open ponds is a common occurrence, however proper pond management can keep contamination under control. The most effective technique to combat contamination is to inoculate open ponds with high algae concentrations (~ 0.5 g/L AFDW). The use of closed PBRs to readily provide dense inoculums will help ensure timely delivery of healthy dense inoculums for open ponds.

2.1.2 Growth Requirements

Growing algae is a systematic scaling of culture volume to the open ponds by diluting dense culture with nutrient rich media and again grown to a higher density. Cells have to be kept in their exponential phase, where they experience optimal growth rates, while nitrogen and iron are monitored by colorimetric assays along with pH to maintain the optimal growth environment. Once the target cell density has been reached in the final culture volume of large outdoor ponds, the culture can be split into two streams: algae stream to be stressed to produce lipids and another to be diluted into new inoculums. Maintaining optimal growth conditions is critical as growth rates can decrease with culture age, less light utilization, and the increase in exposure to

contamination in open ponds. Controlling growth conditions should be managed by monitoring and manipulating the cultures media content.

Algae nutrient media is one of the most important factors to optimal algae growth and oil production (Shelef et al., 1980). Nutrients are the first line of defense against contamination by deterring bacteria with high salt and pH. The correct level of nutrients intracellular and dissolved can trigger byproduct production with certain ions leading to increases in protein, carbohydrate, or lipid synthesis. Nutrients in solution can also be used to harvest the algae by forming densely charged positive ionic species that bind to the negative algae cells, causing flocculation. These nutrient "tricks" are closely held proprietary secrets that drastically reduce production cost by eliminating pesticides, reducing time intensive stressing, and eliminating toxic chemical flocculation. Common macronutrients in algae media include C, N, P, K, S, Mg, and possibly Ca, and are all required at mg/L and higher levels. Phosphorus is highly used in production of DNA and RNA, as well as ATP synthesis, cell wall function, and protein production. It is also a key chemical in auto-flocculation harvesting processes by forming calcium, or magnesium, phosphate complexes with hard water sources, which rapidly flocculate algae from suspension. Potassium is critical to the plants NPK ratio, and is used in enzymes and protein synthesis. Micronutrients include Ni, Zn, B, Vn, Co, Cu, and Mb, and are all required in micro, nano, or picograms per L levels since ions such as copper are toxic at higher levels.

Carbon constitutes 50 % of the algae dry organic content, and is either provided inorganically by CO_2 gas, or by the dissolved bicarbonate ion HCO_3^-, which is absorbed

by the cell and converted to CO_2 (Chen et al., 2009). CO_2 and bicarbonate addition will lower the culture pH and provide buffering capacity. To reduce the high cost of carbon the waste CO_2 from ethanol plants, cement factories, biogas, and spring water rich in bicarbonate can be used. Some nutrient can be inhibitive to carbon dosing, such as calcium, which can lower bicarbonate solubility by binding to and precipitating the bicarbonate out of solution. Calcium can be removed with a polystyrene resin, also known as water softening, to increase bicarbonate solubility.

Nitrogen, which comprises about 10 % of the cells mass, is required for cell growth in the form of nitrate, ammonia, or urea (5 – 50 mM) (Becker, 2008). Nitrogen assimilation can be accelerated by zinc, manganese, or boron deficiency, but is generally assimilated faster than it can be used. Nitrogen deficiency leads to carbohydrate production, and then to lipid production for energy storage. Nitrogen is a growth limiting nutrient which can be analyzed with colorimetric assays to dose growing cultures or to signal transfer of nitrogen deficient cultures from PBRs to stressing ponds (Becker, 2008).

Nutrients represent one of the highest production costs for algae cultivation. Many efforts have targeted unconventional sources of nutrients that can be used to reduce operation costs. Sea water, or brackish water, is the best example as a low cost source of salt and trace minerals that have been shown to increase productivity over pure NaCl. Waste cow urine is high in urea, an organic nitrogen source, and acidic to buffer algae naturally rising culture pH. Lastly waste fermentation effluent can be mixed at

levels of 1 - 5 % (w/w), but should be filtered to remove brown and yellow coloring that can shade the culture (Becker, 2008).

2.1.3 Stressing Algae for Lipid Production

Algae with extended growth in nitrogen deficient environments begin to store carbon source in the form of carbohydrates and lipids to be used as a heterotrophic carbon source when cells can no longer fix carbon from CO_2 by photosynthesis. The process of triggering byproduct synthesis by nitrogen deficiency is called "nitrogen stressing". For optimum oil recovery algae must be grown to high density, then stressed by nitrogen starvation, and then harvested at the peak of their lipid production before the cells begin using the accumulated lipids as a carbon energy source. Sun stressing also accelerates lipid production by diluting algae cultures with nitrogen deficient media and then exposing them to high temperature and solar radiation in shallow ponds to denature carbohydrate synthesis enzymes.

2.1.4 Contamination

Algae are very prone to contamination because of their aquatic environment, growth in hot areas, and production of organic secretions (Becker, 2008). Contamination generally occurs because of decreased cell health and growth, leaving unused nutrients exposed to contaminating microbes. The decrease in cell health and growth is typically due to variation from the cells optimal growth conditions. Cell health should be the primary goal during growth for production standards and contamination

control. Factors such as low pH, low ionic conductivity, insufficient mixing, overgrowth, rodents, media recycling, and the use of waste influent can all attribute to contamination.

Contamination most commonly comes in the form of other algae species and can be ignored if occurring at low levels with nontoxic species like diatoms, which do not decrease end product content or quality (Becker, 2008). The presence of invasive algae species can arise from low pH, high nitrogen, or insufficient mixing. Undesirable contaminants that must be prevented from growing include mold, fungi, bacteria, viruses, zooplankton, rotifers, insects and small mammals like rats and birds. To deter contamination, methods such as high density inoculation, periodic pond cleaning, high salt, high pH, constant mixing, consistent harvesting, and lower nitrogen concentration in the final PBR can be used. Furthermore, it is recommended that growth does not exceed 70 % of the highest possible density to keep cells healthy and in the exponential growth phase. This may be difficult for industrial growth operations to implement. If zoo planktons are detected, the culture should be acidified to pH 3 for 2 hours and then neutralized. To deter insects, the culture can be filtered through a fine mesh screen every other day to remove insect eggs, which can hatch 2 days after being laid. More drastic measures such as fungicides and pesticides can be used such as Benomyl, Antracol, and Kerothane (liq.), which are all agricultural pesticides effective in cultures at 1 mg/L.

There are very few studies on contamination, especially about bacterial contamination in algae biomass. The FDA has rigorous standards for human

consumption of algae which includes standards of only one rodent hair or at most 300 unidentified insect fragments allowed per 100 g of *Spirulina sp*. (Becker, 2008). Contamination can originate from both upstream and downstream sources used in algae processing. Algae farms for biofuels will use water in arid regions where farming is present, and can be rich in bacteria such as *E. coli sp., Salmonella sp., Shigella sp., Leptospira sp., and Staphylococcus sp.*. Water treatment such as denaturing bacteria DNA by UV treatment holds promise. Downstream processing is an even greater source of contamination as biomass concentration steps bring algae cells and secreted organics together allowing microbes to quickly grow and spoil harvested culture if not dried and stored correctly. Biomass should be stored with less than 7% moisture content in the possible presence of preservatives and CO_2 or N_2 gas.

2.2 Current and Potential Uses of Microalgae

The original focus of algae research was to develop a super-crop for low cost high protein production and general nutrient substitute for third world production. Commercialization of algae potential as a super-crop has been limited by various technological and political factors, including high production cost and safety concerns when used for human consumption (Becker, 2008). Alternative uses of algae for their nutritional and/or energy content have been developed to include ruminant and aquaculture feed, high value nutraceuticals and omega-3 fatty acids, skin therapy extracts, environmental remediation products, and chemical byproducts. Application of

microalgae for biofuels production is currently being investigated and developed by industry and academia.

2.2.1 Animal Feed

Algae have been regularly used as a protein source for ruminants and larger sea organisms in many countries. Cows, sheep, and pigs have all been extensively studied by feeding them digestible algae species, C*hlorella* or *Spirulina,* mixed with a combination of fillers, including alfalfa, oats, and hay to dilute algae's valuable high protein content. These toxicological studies have not shown deleterious effects on the animals, and even showed a higher weight gain due in part to the absorption of algae's omega-3 fatty acids (Certik and Shimizu, 1999). Common algae species are not easily digested by humans because of the strength of the algae cell wall. Species such as *Chlorella vulgaris* and *Spirulina sp.* can be digested and consumed by humans because of their easily broken cell wall. The use of algae as ruminant feed originated from the century old practices of using algae as live feed for cultivating complex sea-life such as crustaceans, oysters, scallops, clams, mussels, and fish (Graham and Wilcox, 2000). Specialty lipids, such as docosahexaenoic and eicosapentaenoic acids (EPA and DHA), are derived from algae-fed fish, which store the lipids in muscle cells making oil extraction more economical compared to extraction from plant cells (Jiang et al., 1999).

2.2.2 Wastewater Treatment and Environmental Remediation

The fast growth rate of algae makes it an attractive microbial species for several industries including environmental remediation. Algae are known for their high rates of nutrient uptake including toxic metals, aluminum, iron, and heavy metals like mercury, copper, cadmium, lead, and arsenic (Becker, 2008; Canizares-Villanueva et al., 2001; Chen and Jiang, 2001). To remediate contaminated waters, algae can either be grown in the contaminated source, or added to the contaminated source as an absorptive powder. It only takes 24 hours to saturate the algae with metals from the contaminated waters, at which point more algae can be added, or the algae and absorbed toxic metals can be removed and the process repeated (Becker, 2008). The ability of algae to absorb and metabolize ionic toxins also makes algae a popular organism for producing radio labeled bioproducts (Becker, 2008). Algae byproducts can be radio labeled with isotopes which can be absorbed and converted into valuable bioproducts such as radio labeled lipids, proteins, or carbohydrates. This trait not only holds promise for treating environmental radiation contamination but can be regularly used for studies on synthesis and bioconversion of radio labeled bioproducts that can then be traced in physiological and or toxicological studies (Becker, 2008).

Additional remediation of contaminated waters includes the treatment of municipal wastewater (Narasimhan, 2010). Wastewater treatment is already possible by the use of pre-existing processes including bacteria denitrification and clarification. However, conventional wastewater treatment is cost negative, requiring energy input to treat influent with little to no marketable byproduct. Valuable microalgae can be grown

on the high concentration of nutrients in wastewater, while treating the wastewater in place of more expensive treatment processes by biologically converting harmful nitrogen organics, like ammonia, into cellular biomass (McGarry and Tongkasame, 1971). The biomass can then be removed and then used, sold, or converted into a high value byproduct (Narasimhan, 2010). Algae can also be used in denitrification wastewater steps in place of traditional treatment processes. The biomass can be used as an animal protein source, but would be unsafe for human consumption given its growth on waste influent (McGarry and Tongkasame, 1971). There has been extensive work on algae for wastewater treatment, unfortunately though, the residence time required for de-nitrification by algae compared to using conventional bacteria has been a limiting factor in commercializing the technology.

2.2.3 Nutraceutical and Pharmaceutical Products from Algae

Specialty byproducts from algae include protein, vitamins, pigments, and other nutraceuticals (Becker, 2008). More valuable proprietary byproducts from algae include hormones, waxes, sterols, lubricants, amino acids, enzymes, polysaccharides, hydrogen gas, biogas, specialty fatty acids, and recombinant therapeutic biomolecules (Becker, 2008). The production of therapeutic chemicals and proteins from algae has the potential to become an economical production platform in the nearly trillion dollar pharmaceutical industry (Rasala et al., 2010).

Natural therapeutics from algae are able to combat a variety of infections from cancer to HIV. For example, several brown algae strains produce a powerful anti-

oxidant, which can trigger apoptosis in human leukemia and colon cancer. The polysaccharides of algae can be highly sulfated, and are inhibitive to the reverse transcriptase enzymes of HIV (Barsanti and Gualtieri, 2006; Gustafson et al., 1989). Some algae biomolecules, such as *Spirulina*'s enzymatic hydrolates can treat ulcers, burns, and non-healing wounds (Safar, 1975). Therapeutic recombinant proteins and other recombinant biomolecules hold the greatest potential for algae biotechnology. Proteins such as erythropoietin, interferon β, and insulin are all native human proteins that can be expressed in the chloroplasts of *Chlamydomonas reinhardtii* (Rasala et al., 2010). *C. reinhardtii* is one of a few choice strains used for genetic modification. Algae offer low cost, robust cellular growth, and rapid scalability, and can easily be grown in sealed bioreactors like mammalian and *E. coli* cells, reducing the possibility for gene flow that could be more prolific in open air ponds. The projected cost of producing transgenic soluble protein from algae, according to early studies, is close to the least expensive production system of only $0.60 per gram of soluble protein (Dove, 2002; Rasala et al., 2010). Therapeutic expression levels and purification methods are currently being developed to help make algae pharmaceuticals economically attractive.

2.2.4 Biofuels from Algae

The high lipid and carbohydrate content of microalgae makes them excellent feedstock for biofuels since biodiesel, gasoline, and ethanol can all be produced from algal byproducts. Lipids can be chemically converted by transesterification into biodiesel, or cracked into shorter chained hydrocarbons for use as gasoline (Dupain et

al., 2007). The cracking process requires a purer lipid profile than currently produced in algae, but has the potential for improvement with genetic modification and lipid purification (Nikolov, 2010). Carbohydrates can be converted into ethanol by microbial fermentation. However, the recovery of sugars as well as the microbial digestibility of algal polysaccharides, such as cellulose, require further research (Goyal et al., 2008). Genetically modified algae can produce monosaccharides such as glucose and can even secrete it for easier recovery (Nobles Jr., 2010). There are other conversion processes that can produce chemical energy utilizing algal nutritional content (Amin, 2009).

Compared to other biofuel feedstocks in Table 2.2, algae have a significantly higher oil yield. This is possible in part because of algae faster growth rates that result in more biomass produced per hectare per year than traditional biofuel feedstocks (Chisti, 2007).

Table 2.2. Comparison of biofuel feedstocks (Chisti, 2007).

Crop	Oil yield (L/ha)	Percent of existing US crop area[a]
Corn	172	846
Soybean	446	326
Canola	1190	122
Jatropha	1892	77
Coconut	2689	54
Oil palm	5950	24
Microalgae[b]	136900	**1.1**
Microalgae[c]	58700	**2.5**

[a] For meeting 50% of all transportation fuel needs of the United States (2007)
[b] 70% oil (by wt.) in biomass
[c] 30% oil (by wt.) in biomass

2.2.5 Biorefinery Concept of Algae Utilization

Despite the value of algae biomass there are still several capital investment intensive factors that have to be taken into considerations. These include open pond structures, closed photobioreators and processing equipment for harvesting, biomass concentration, and oil extraction. In order to build investment support for producing low value products from algae, an attractive risk-to-return ratio must be developed by utilizing every valuable product from the cultivation process to increase overall revenue and decrease energy input. The separation and conversion of algae nutritive and energy content into profitable byproducts and reusing nutrients and excess heat from conversion processes can be done through a concept referred to as "algae biorefining" (Singh and Cu, 2010). The envisioned algae biorefinery would be similar to an oil refinery by producing fuels and chemical byproducts while recycling as much waste streams and latent energy as possible (Figure 2.1).

Existing biorefineries offer possible integration of pre-existing processing steps, such as centrifugation and solvent or protein extraction, if a mixed end product does not reduce market value and revenues. The combination of algae and corn biomass fractionation and extraction of carbohydrates, oil, and protein could help finance expensive concentration and extraction equipment for algae processing, and would also give financial stability to existing biorefineries by diversifying their commodity input. This stability would help revive ethanol plants which have struggled with the increase in the cost of corn commodities, and the uncertain future of government ethanol subsidies. Such process integration could hopefully be achieved without drastically changing the

processing steps used with corn byproducts, and may increase product revenue with the inclusion of algae's specialty EPA and DHA lipids. In addition, dry and wet milling byproducts such as DDGS and corn germ are similar to several algae species and generic defatted algal biomass in terms of high protein and starch content and moderate lipid concentration (Belyea et al., 2004; Widmer et al., 2007). This similarity makes these byproducts ideal candidates for process integration with algae biomass.

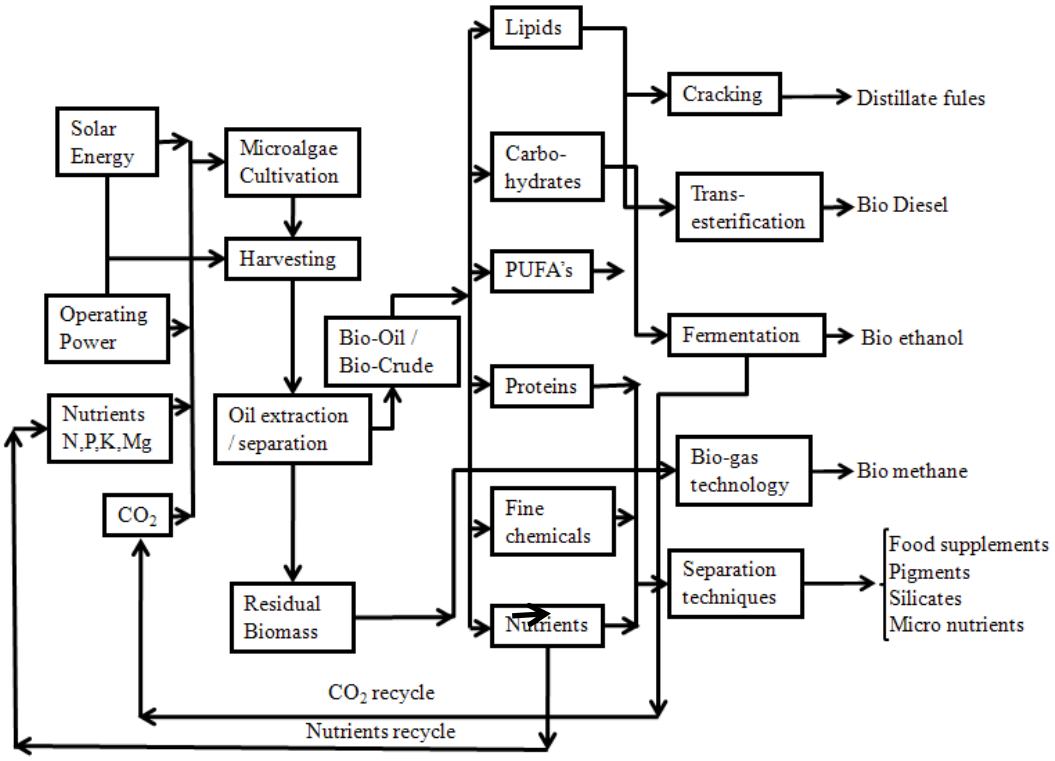


Figure 2.1. Process diagram of a model algae biorefinery. (Singh and Cu, 2010).

2.3 Harvesting and Concentration Methods

The economic feasibility of microalgae production is largely dependent on the initial capital and operating cost of the harvesting technology used to concentrate dilute algal suspensions into denser slurries, making the biomass more suitable for further processing. The harvesting and drying processes combined can account for up to 30 % of the end product cost (Gudin, 1986). Harvesting and dewatering of algae economically is not a single step process. Several steps are typically required to reduce algae moisture content to less than 7 % to deter spoilage and allow for long term storage (Chen et al., 2009). The harvesting step depicted in Figure 2.1 could consist of multiple concentration steps and a separate drying step. Typically, complete dewatering would require two steps, a low-cost pre-concentration harvesting step to reduce process volumes, and a second higher energy post harvesting concentration step to further concentrate the biomass. The objective of the harvesting step is to remove at least 90% of water from the algae culture at high efficiency and low cost. The objective of the possible post harvesting concentration step is to increase the biomass solids content above 15 % before drying. Drying can then be used to yield a 7 % moisture content as seen in Figure 2.2 (Shelef et al., 1984). The characteristics (throughput, cost, maintenance, etc.) of potential harvesting technologies for microalgae will be reviewed below.

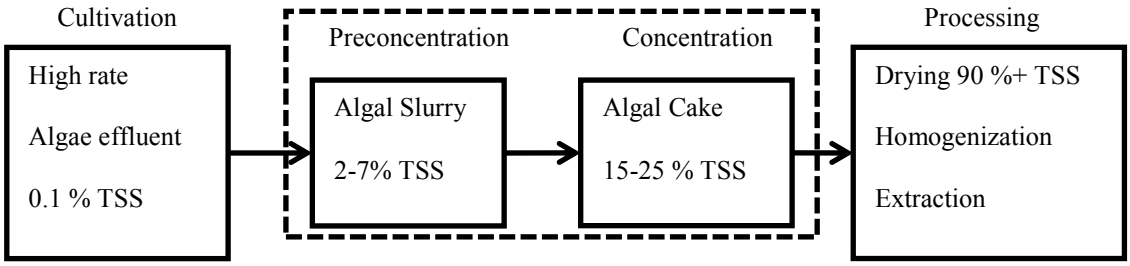


Figure 2.2. Block diagram of algae growth, harvesting, and processing (Adapted from (Shelef et al., 1984)).

2.3.1 Centrifugation

Centrifugation often serves as a bench mark to compare all other concentration techniques because it is the quickest and most effective process for the removal of particles from suspension without the aid of chemical additives. Centrifugation could be a rather expensive harvesting method because of the use of energy inefficient electric motors and high capital cost. This high capital cost and energy requirements of centrifugation create an economic bottle neck preventing its use for algae harvesting. Another potential downside of centrifuges is the existence of large shear forces during centrifugation that can lyse fragile cells and release algal oil into the dilute medium, thus, making oil recovery difficult if not economically impossible (Molina et al., 2003). Centrifuges could serve several different purposes including initial algae harvesting, post harvesting concentration, and byproduct recovery (Becker, 2008).

Only a few centrifuge types can quickly process large volumes of dilute microalgae culture. These include disc-stack, scroll discharge (decanter), and tubular bowl centrifuges (Richmond, 2004). Disc-stack is a more commonly used centrifuge as

it is effective, continuous, can be modified to optimize particle removal and consumes relatively 1 kW*h/m^3 (Mohn., 1980). Scroll discharge and tubular bowl centrifuges are also attractive and can yield algal slurries with lower moisture content than disc stack centrifuges (Letki, 1998; Mohn, 1988; Shelef et al., 1980).

Disc-stack centrifuges can easily process large volumes of unprocessed dilute culture, and can be run semi-continuously for several days. They can concentration 0.1 % (TSS) algae cultures to 2 % (TSS) after a single-pass. Subsequent passes through the centrifuge can yield concentrations as high as 12% TSS (Mohn, 1988). Scroll discharge, also known as decanter centrifuges, require a higher algae input concentration of 1.5 - 2 % (TSS) and yield a cheese-like product with 25 % (TSS) (Mohn, 1988). This qualifies decanter centrifuges as a post harvesting concentration step since decanter centrifuges require input slurries of 1.5 - 2 % (TSS). Tubular bowl centrifuges combine the benefits of both disc stack and decanter centrifuges with its ability to process dilute cultures to a high solids content of more than 25 % TSS (Letki, 1998). However, tubular centrifuges are impractical (low throughput capacity) for large scale microalgae concentration, but are a good candidate for concentrating genetically-modified algae species expressing pharmaceutical products due the tubular centrifuges high sterility features (Letki, 1998).

Several algae species can be efficiently removed from their suspensions by centrifugation due to their larger size and greater tendency to coagulate and settle. The use of centrifuges for larger-size species, such as *Spirulina sp.* and *Dunaliella sp.,* is more economical because it requires lower energy, or G forces (Becker, 2008; Mohn, 1988). A possible solution to microalgae's small size and slow settling rates is to use of

pre-concentration methods such as flocculation to increase the microalgae size by forming larger aggregates that can be easily removed by centrifugation.

2.3.2 Filtration

The filtration of large volumes of dilute microalgae cells by dead end or depth filtration can make many membrane concentration processes economically unattractive due to required capital cost, maintenance, and low throughput (Becker, 2008). Membranes that are economical and effective, with high cell recovery and acceptable throughput rates, have pore diameters larger than 20 μm (Becker, 2008). Larger pore membranes are commonly used for commercially grown filamentous algae *Spirulina* or aggregated microalgae (Becker, 2008). Algae such as *N. oculata* and *C. vulgaris* have cell diameters close to or less than 5 μm and would easily pass through these membranes or foul them. Dead-end membrane technology could serve as a post harvesting concentration step during microalgae harvesting (Mohn, 1988; Narasimhan, 2010). Dilute microalgae form gel-layer on the membrane surface and eventually fouls it instead of forming a cake like structure that allows for efficient filtration (Becker, 2008; Harrison, 2003). Harvesting algae to a higher total suspended solids may encourage aggregation which will allow the use of large-scale filtration devices such as filter presses, belt filters, or vacuum drum filters (Shelef et al., 1984).

Belt filters can be used to filter dilute microalgae due to their continuous filtering process. The belt filter capacity is dependent on algae size; filtration of *Chlorella* and *Oocystis* had a throughput of 2-3 m^3*h^{-1}, whereas larger algae such as *Spirulina sp.*,

Micractinium sp., or S*cenedesmus sp.*, greater than 200 m^3*h^{-1} (Becker, 2008; Dodd and Anderson, 1977).

Vacuum drum filters are similar in design, but require a pre-concentrated slurry of 2% TSS (Becker, 2008). The vacuum drum filter is coated with a pre-coat filter aid, which is commonly comprised of starch, cellulose, or even paper fibers (Dodd, 1979; Harrison, 2003). The use of filter aid allows vacuum drum filters to effectively harvest microalgae by deterring pore blockage and fouling by forming a filter cake on the filter surface (Harrison, 2003). The filter is dipped into the pre-concentrated culture where the algae slurry is caked onto the filter with the help of the filter aid and vacuum pressure (Becker, 2008). The algae slurry is then vacuum dewatered and scraped off the filter with care as to not damage the drum filters pre-coat, or to contaminate or dilute the recovered biomass with excess filter aid (Harrison, 2003). Continuous filters like the belt and vacuum drum filters can also be used to further dry harvested algae cake combining the harvesting and drying processes.

The concept of using filter aids is an effective method for helping microalgae cake onto the filter surface without plugging the filter pores. The precoat filter aid can be toxic and limit the usage of the harvested biomass if higher than 15 % (w/w) concentration in the harvested biomass (Becker, 2008). Other conventional filter aids such as diatomite could disallow the use of algae as feed, since diatomite is made of the skeletal remains of diatom algae, which are high in silica and have a history of producing toxins (Harrison, 2003).

2.3.3 Flocculation

The high capital investment for centrifugation and the low throughput rates of membranes remain the greatest hurdle for their use as initial harvesting processes. To negate these hurdles, a cell aggregation process can be used to increase microalgae size and settling rate, while reducing processing volumes for more economical post-harvest concentration or processing. Flocculation can take place by chemical flocculation, auto-flocculation, bio-flocculation, or microbial flocculation. Chemical flocculation uses salts or charged polymers to form flocks, while the reportedly unreliable process of auto-flocculation involves the pH initiated precipitation of charged metallic ions dissolved in the growth media to cause flocculation (Becker, 2008; McGarry, 1970; Sukenik and Shelef, 1984). Bio-flocculation refers to species, unlike *N. oculata*, that can experience a reduction in their cell wall charge commonly occurring during the cells stationary growth phase (Oh et al., 2001). Lastly, microbial flocculation is an unconventional method that contaminates algae cultures with bacteria that secrete polymers causing flocculation (Lee et al., 2009).

Flocculation involves the aggregation of two or more cells caused by cell-cell attraction or by chemical intermediates such as polymers and ions. Cells share the same negative charge, which causes them to repel each other like magnets of the same polarity. In order for flocculation to occur, the cells have to overcome their repulsion for each other, and approach each other close enough for attractive Van der Waals forces to cause an attraction (Henderson et al., 2008b). Flocculation entails the process of multiple cells joining together to form an aggregate, or flock, that can be removed from a

suspension by forces such as gravity sedimentation, flotation, or filtration (Aleman et al., 2007). Several factors that encourage flocculation include Brownian motion, extracellular organic material, cellular metabolism, ionic strength, and dissolved oxygen content (Becker, 2008; Henderson et al., 2008b). Factors that inhibit flocculation may involve hindrances caused by algae filaments, spines, or flagella, as well as temperature and pH (Becker, 2008; Henderson et al., 2008b).

To understand the difficulty of forming flocks, Figure 2.3 displays the amount of potential energy required for two particles of the same charge to aggregate, and how a high ion concentration between the particles decreases the amount of energy required for cells to aggregate (Pieterse and Cloot, 1997).

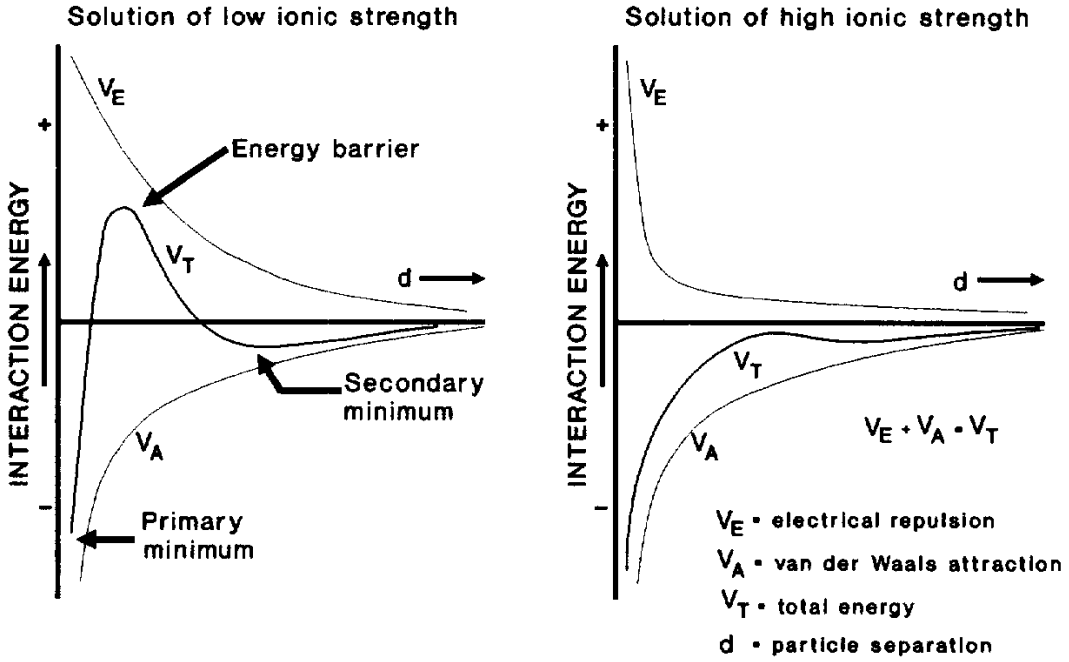


Figure 2.3. Potential energy between colloidal particles vs. the distance between them with low and high ionic strength (Pieterse and Cloot, 1997).

The foremost effective tools available to study and develop flocculation processes are jar test and zeta potential measurements. Jar tests, also called settling tests, measure the amount of shear required for flocculant mixing and efficient aggregate formation while providing standard parameters to observe and measure settling rates. Zeta potential measurements characterize the electric potential or repulsive forces, around the algae cell. These useful concepts and tools were employed to develop the *N. oculata* flocculation and will be reviewed below. There are two principle chemical flocculation methods, ionic flocculation and polymer flocculation.

2.3.3.1 Ionic Flocculation

Ionic flocculation uses positively charged ions to reduce algae's negative charge towards neutral (0 mV), and cell-cell repulsion that stabilizes algae suspensions. The amount of charge reduction or charge neutralization required for optimum flocculation is species dependent and can be targeted based on a cell charge density dependent (Henderson et al., 2008b). To destabilize a microalgae suspension multi-valent ions such as tri-valent cationic metallic species of aluminum and iron are necessary for efficient flocculation because of low cell concentrations (0.1% TSS), strong negative charge, and the biological ability of algae to "repair" cell wall charge reduction when exposed to counter ions (Clasen et al., 2000).

Ionic flocculation has several negative issues associated with its use. The toxicity of ionic flocculants limits their usage for feed applications and has led to efforts

to remove flocculants from the biomass in post harvesting processes (Molina et al., 2003). Aluminum bound to algae can possibly be removed by solubilizing with dilute HCl or EDTA solutions (Volesky, 1990). This process could take part during algae harvesting by spraying caked algae, fixed to a rotating drum filter, with dilute acid.

Ionic flocculation is sensitive to pH, with an effective aluminum dosage of 150 mg/L $AlCl_3$ requiring a pH of 6.5. Better flocculation was observed from pH 5.3-5.5, with a lower dosage of 80 mg of $AlCl_3$. $FeCl_3$ dosing was lower at 50 mg $FeCl_3$ but required additional acidification to an optimum pH of 3.5 (Becker, 2008). The pH adjustment was critical for flocculation to occur as pH levels higher than 7 saw no flocculation.

Ionic flocculation efficiency and speciation is highly sensitive to culture pH. The culture pH dictates the dissolved concentration and ionic species formed by hydrolysis (Gregory and Duan, 2001; Hayden, 1974; Xiaoying et al., 2009). The sensitivity of flocculation to pH comes from ionic species hydrolyzing at acidic conditions and is detailed in aluminum ion solubility curves (Figure 2.4). The range of positively charged aluminum species in Figure 2.4.b that are soluble is greater at a higher pH than those of iron (Figure 2.4.a). This gives direct evidence to the economic advantages of aluminum over iron with less acidification required for optimum flocculation (Becker, 2008).

To better explain the change in aluminum species with changing pH and understand the mechanisms responsible for flocculation, aluminum solubility curves in Figure 2.5 must be examined because of its higher $AlCl_3$ concentration. Figure 2.5 is similar to Figure 2.4, but instead shows the dosing effect on aluminum species solubility

vs. pH. Flocculant concentrations used in this study exceed those in Figure 2.4.b, but would still yield a similar solubility curve with vertical dissociation curves due to higher $AlCl_3$ dosage. According to Figure 2.4.b, the pH of Al^{3+} formation by hydrolysis reaction occurs between pH 4 and 5, which correlates to the report by Bernhardt (1986) who listed Al^{3+} formation at pH 4.3 (Bernhardt et al., 1986; Duan and Gregory, 2003; Letterman, 1999).

(a)

(b)

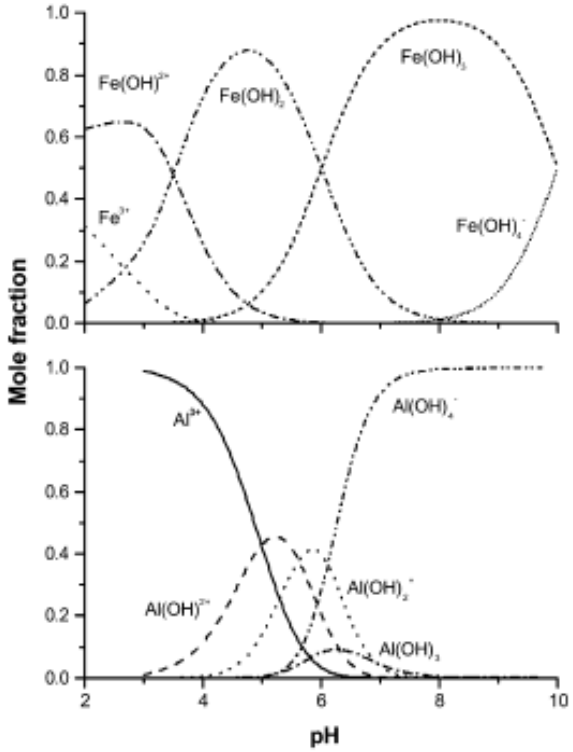


Figure 2.4. Effect of pH on hydrolyzing aluminum and iron ionic species (Gregory and Duan, 2001).

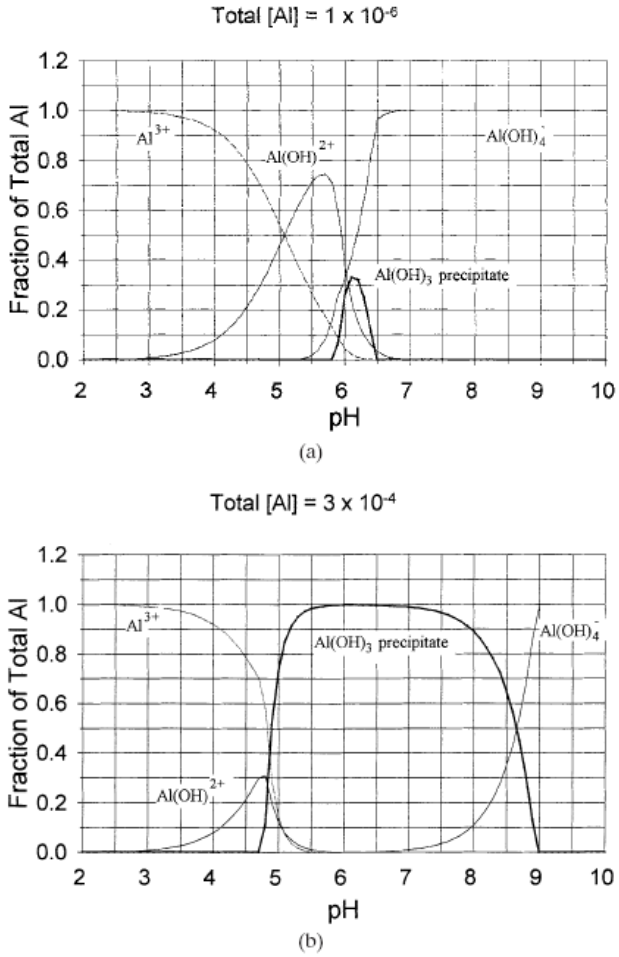


Figure 2.5. Aluminum solubility curves showing the correlation between pH and aluminum hydrolysis, (a) $1X10^{-6}$ M Aluminum, (b) $3X10^{-4}$ M Aluminum (Letterman, 1999).

The formation of Al^{3+} species at pH 4.3 from the neutral aluminum hydroxides present above pH 5 in Figure 2.5 is counter intuitive to the process of coagulation by charge neutralization since optimum flocculation was listed at pH 5.5 (McGarry, 1970). Cationic Al^{3+} should cause neutralization of negatively charged cells, while neutral aluminum hydroxide precipitation should have no effect on cell wall charge reduction. Data in Figure 2.4 is an incomplete representation of the actual situation because it

discounts the formation of cationic poly-aluminum hydroxide species (PACl). PACl, which is commonly used for flocculation and can randomly form when solubilizing aluminum water, can form polyionic aluminum species with valence charges higher than 3+. This hypothesis is well supported by Shelef et al., (1984), who stated that *"The effects of ferric and aluminum salts are brought about by their hydrolysis products and not by the simple aqua-metal ion themselves*", (Shelef et al., 1984). The formation of aluminum hydrolysis products produced is shown by Shelef et al., (1984) in Figure 2.6.

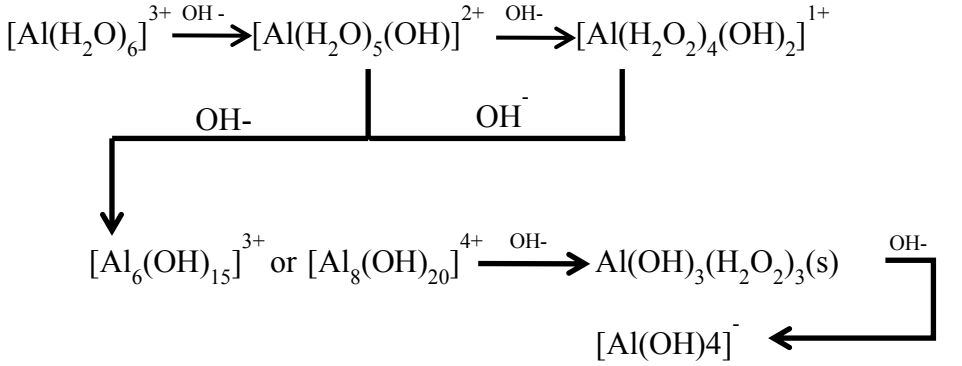


Figure 2.6. Stepwise oxidation of aluminum ion species with increasing pH (Shelef et al., 1984).

The aluminum solubility graphs of Figures 2.4 and 2.5 do not show the presence of these polyionic species and can be misleading as to which ions are mainly responsible for initiating flocculation. To support aluminum hydroxide polyionic species initiating flocculation instead of pure aqua metallic Al^{3+} species, the empirical formula for calculating the minimum flocculant dose required to initiate coagulation (C_i) is examined.

$$(C_i)_{flocc} = \frac{9.85 \times 10^4 \varepsilon^3 k^5 T^5 \gamma^4}{\mathcal{N} e^6 A^2 z^6} \quad [2\text{-}1]$$

The variable of greatest interest in equation [2-1] is the counter ion charge number (z^{-6}) due to its strong effect on reducing flocculant concentration with increasing counter ion charge number. All other variables are fixed for the equation. The counter ion charge is the ionic charge of the flocculant present in solution: +2 for $Al(OH)^{2+}$, +3 for Al^{3+}, and 4+ for $Al_8(OH)_{20}^{4+}$. From equation [2-1], assuming all other variables consistent, the $(C_i)_{flocc}$ ratio for 2+, 3+, and 4+ species would be 64:11:1. Thus $Al_8(OH)_{20}^{4+}$ species would initiate coagulation at lower electrolyte concentration than Al^{3+} or $Al(OH)^{2+}$. Penta-valent aluminum species $Al_3(OH)_4^{5+}$ is also a commonly observed aluminum hydrolysis product that would flocculate under even lower concentration levels (Meghzili et al., 2008). Since the concentration and type of $AlCl_3$ hydrolysis products depend on pH (Figures 2.3 and 2.4), a shift in coagulation efficiency as a function of culture pH should be expected.

Direct aluminum charge reduction was measured with organic waste water by the addition of 0.06 g/L of aluminum sulfate (Alum) in Figure 2.7. Optimum color reduction occurred between pH 5 and 6, where the colloid zeta potential was reduced from about -15 mV at pH 7 to -12, -5, and -0.5 mV for pH 6, 5, and 4.5, respectively. Removal efficiency was reversed at pH 4.5 before reaching the turbidity level seen near pH 7 where no flocculation occurred. There was however a charge reduction of about -

7.5 mV observed near pH 4, despite the absence of flocculation. This amount of charge reduction is counter-intuitive since pH 6 had a zeta potential of -12.5 mV and yielded a removal efficiency equal to that of pH 5, which had a zeta potential of about -7.5 mV.

The only explanation for having a high removal efficiency at pH 6 at zeta potential of -12.5 and no flocculation at pH 4 at of -7.5 mV is that semisoluble charged ionic hydroxide, such as $Al_{13}O_4(OH)_{24}^{7+}$, was capable of bridging cells into flocks (Xiaoying et al., 2009). Some of the aluminum hydroxide species that could have formed to cause Alum flocculation of the wastewater in Figure 2.7 include $Al_6OH_{15}^{+3}$, $Al_7OH_{17}^{+4}$, and $Al_8OH_{20}^{+4}$ (Ravina, 1993). It was noted by Ravina, (1993), that Alum's anion SO_4^{-2} can be incorporated into the metallic hydroxide, and can inhibit its flocculation efficiency by reducing the hydroxides charge. The Cl^- anions of $AlCl_3$ are less likely to be incoporated into a poly-nuclear ionic species since the Cl^- anion is less negative than SO_4^{2-}, and PO_4^{2-}, which can also be precipitated and bound to aluminum at neutral and basic pH levels (Sukenik and Shelef, 1984).

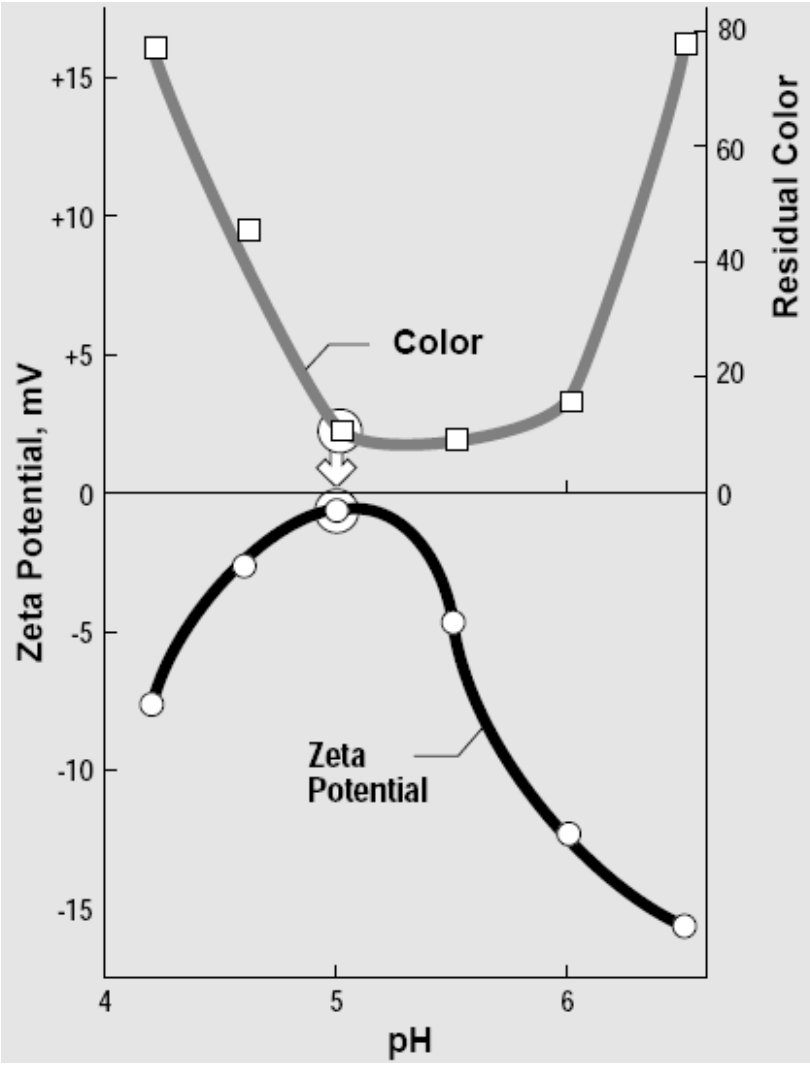


Figure 2.7. The effect of pH on zeta potenital and supernatant tubidity with the addition of 0.06 g/L Alum flocculant (Ravina, 1993).

Finally, to account for biological factors that can affect metallic charge neutralization, an inorganic quartz particle colloid was flocculated with Alum flocculant in Figure 2.8. Similar to Figure 2.7, the colloids electrophoretic mobility, which is directly related to zeta potential, was reduced with acidification until about pH 4.5. The electrophoretic mobility between pH 4.5 and 4.0 reduced from 1.5 to 0

((μm/sec)/(V/cm)) because of a hydrolysis shift to lower-valence aluminum species. Quartz particles make an excellent control for testing aluminum flocculants without the presence of biological variables such as cell age, EOM, and algae ability to repair cell wall charge reduction (Clasen et al., 2000). Additionally, quartz particles are highly negatively charged and have a low isoelectric point at pH 2, which will not interfere with the flocculation pH range tested (Somasundaran, 2006).

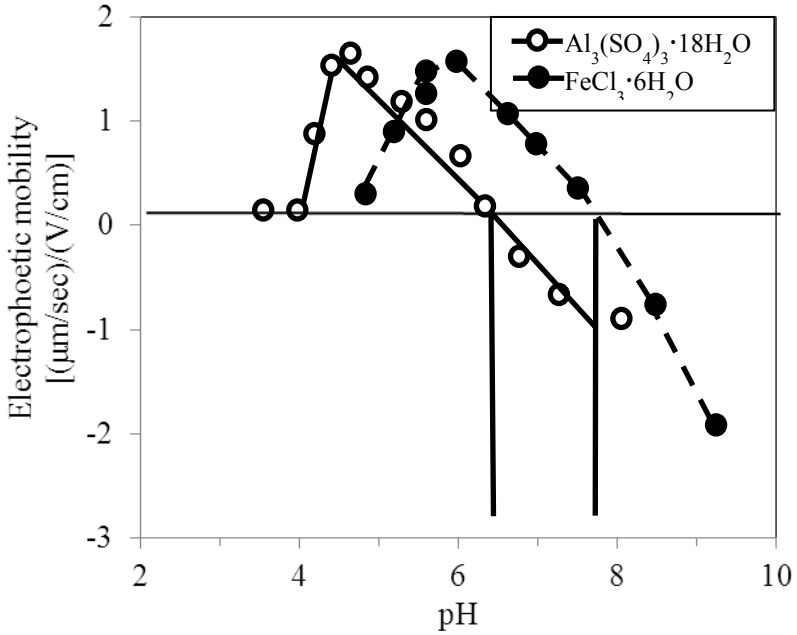


Figure 2.8. The effect of pH on the electrophoretic mobility of quartz particles with $Al_3(SO_4)_3*18H_2O$ flocculant (Bernhardt and Clasen, 1991).

2.3.3.2 Polymeric Flocculation

Polymeric flocculation involves the use of long chain charged polymers that are able to bind two or more algae cells by bridging the μm length gap between the cells,

forcing them to aggregate into dense flocks capable of settling. Polymers are also able to reduce a particles zeta potential depending of the polymer charge density (Uduman et al., 2010). Positively-charged cationic polymers, such as polyethylene amine, show the highest binding efficiency as the positively charged groups bind to the negatively charged algae cells (Shelef et al., 1984). Polymers offer an effective solution to the sometimes difficult process of flocculation, since many polymers are not pH sensitive like ionic flocculants. Polymers also form large flocks that settle in seconds, versus the slow settling rates that can be associated with ineffective ionic, auto, or bio-flocculation.

Polymeric flocculation process can be inhibited by the high conductivity of brackish and salt water. The high-salt levels inhibits polymer flocculation by reducing the charge density and the reduce repulsion between charged groups on the polymer that keeps the polymer extended to its full length to effectively bridge algae cells (Bilanovic et al., 1988; Bolto and Gregory, 2007). The ionic polymer will randomly coil to a smaller size that is still able to bind charged algae cells, but is unable to link or entrap several cells together to form a flock. The effect of ionic strength on the conformation of a charged polymer is depicted in Figure 2.9. Salt inhibition can be minimized by using polymers with rigid backbones, or by extended polymer mixing times in the high conductivity solution during polymer preparation before flocculation (Morales et al., 1985).

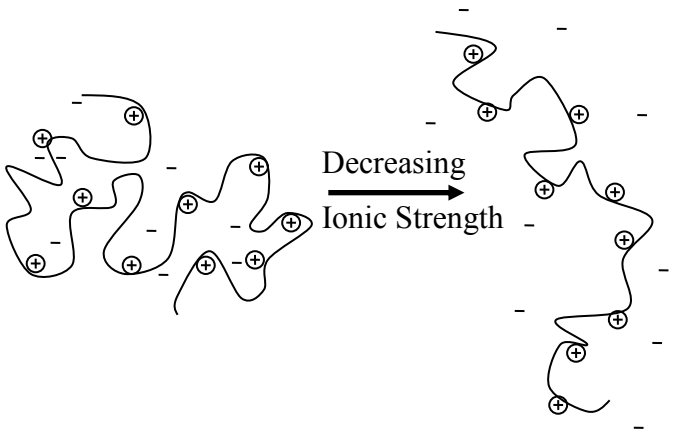


Figure 2.9. The expansion of a polyelectrolyte chain with decreasing ionic strength (Bolto and Gregory, 2007).

pH adjustment can also help with polymer preparation for pH sensitive polymers such as chitosan, where a lower pH will decrease the viscosity of the polymer solution allowing for more homogenous mixing and flocculation during dosing. Flocculation can then be pH triggered by an increase in pH which will increase the chitosan's viscosity in the solution and initiate settling of cellular aggregates (Bilanovic et al., 1988; Morales et al., 1985). Chitosan is a popular flocculant for algae removal in aquaculture industry because of its use as a food additive. A dosage of 45 mg/L of chitosan resulted in 100% removal efficiency of *Chlorella sp.* and *Dunaliella tertiolecta* (Morales et al., 1985).

Synthetic polymers such as polyethylamine and polyacrylamides are commonly used for removing negative particles from dilute solutions. The charge density of polyacrylamides is pH sensitive as ester groups can hydrolyze above pH 6, causing a loss in the polymers charge density (Bolto and Gregory, 2007). In flocculation studies, 10 mg/L of high molecular weight (MW) polyethylamines gave successful clarification (Moraine, 1980; Tilton et al., 1972). Due to proprietary formulation, commercial

polyethylene amines designated as “Zetay 51” and “Dow 21M”, have respective optimal pH ranges of above 9 and from 4 to 7 (Shelef et al., 1984). An increase in a polymers flocculant efficiency is proportional to its size (MW), because the increase in MW increases the polymers charge density and length to bridge algae cells (Tilton et al., 1972). The residual polymers in solution can lead to problems with media reuse (Molina et al., 2003). The recycling of algae media dosed with flocculants can reduce biomass productivity. A 30% decrease in algae growth in chitosan flocculated media was measured (Narasimhan, 2010).

2.3.3.3 Electrolytic Flocculation

Electrolytic-flocculation (EF) has been proven to effectively remove up to 95% of algae in fresh water (Poelman et al., 1997). EF works by both charge neutralization and by encouraging aggregate formation by electrical field forces. In EF reactive electrodes composed of iron or aluminum are placed in a culture and a current is run across the electrodes. The electrodes will oxidize, solubilizing metallic ions, dosing the culture with ionic flocculant. This method may be more effective than adding metallic salts, as the aluminum is not accompanied by counter ions such as Cl^- or SO_4^-. The current causes the negatively charged algae cells to migrate towards the positively charged electrode and enhance cell-cell interactions and aggregation (Uduman et al., 2010). However, concerns arise with the use of EF in salt water as the high ionic strength would decrease the ionic solubility of metallic ions from the electrode source and could increase the current level required to dose the algae with ionic flocculant.

Additional concerns arise with the use of EF including byproduct quality with the application of high current to the suspended dilute biomass (Poelman et al., 1997).

2.4 Factors Effecting Flocculation

2.4.1 Dosage, Mixing Conditions, and Velocity Gradients

Factors effecting flocculation such as dosing, mixing conditions, and particle concentration can all better optimized using the jar test apparatus (Figure A.4.1). The jar test apparatus is a bench scale method that mimics large scale flocculation processes and allows for parameter optimization and scalability before scaling up the flocculation process (Wakeman and Tarleton, 1999). The jar tester gets its name from the standardized "jars" used to flocculate the culture, but is also referred to as a settling test (Wakeman and Tarleton, 1999). Similar processes can be done at small scale, but the scaling of mixing conditions does not apply to these bench scale processes without standardized paddle sizes and velocity gradient (1/s) curves. The ability to optimize the velocity gradient qualifies Jar Testing as the optimum process for determining optimum scalable flocculation parameters. It is important to optimize flocculation at a small scale before attempting the process at a larger scale because of the cost involved and the possible toxicity of flocculant overdosing. Factors such as the mixing speed, also known as the velocity gradient, time of mixing for both fast and slow speeds, as well as the minimum flocculant dosage required for optimum flocculation must be determined with a jar tester (Svarovsky, 1990). The velocity gradient is denoted by the variable G, and is

correlated to the jar tester's propeller speed in the G-curve shown in Figure A.4.2. These G-curves are important as they are the only way to accurately scale mixing conditions. The velocity gradient (G) is computed according to Stokes theory where by $G = W*\mu^{-0.5}$ (Lai et al., 1975).

Optimum mixing conditions including mixing time and rpm, or shear, are crucial to the formation of large flocks for their removal by either settling or flotation. Optimum mixing will encourage cell-cell interactions to increase aggregation and form large flocks, but will not break preexisting flocks by imparting excessive shear (Svarovsky, 1990). A delicate balance must be met between the amount of shear and the time of mixing, as the electric cost of mixing large cultures can be inhibitive to low value product separations.

Furthermore a two-stage mixing process is normally employed to ensure homogenous mixing of the flocculant by fast mixing and optimum aggregation by slow mixing speeds without breaking flocks. Typically a rapid mixing stage will be employed to mix in flocculant and form small flocks for 1 to 3 min. A slow mixing stage is then used to encourage cell-cell interactions and aggregation into larger settling flocks for 15 minutes to an hour (MRWA). Both of these mixing speeds must be optimized to produce efficient flocculation. To optimize the velocity gradient and mixing time previous cited literature cited speeds are normally targeted with visual observation of flock formation or breakage. This comparison along with the measured settling rates helps formulate an optimum velocity gradient.

In the case that optimum mixing conditions are not met, additional flocculant could be required for efficient flocculation. If flocculant dosage is increased to excessive levels a colloid charge reversal can occur, which will stabilize algae cells by reversing their charge from negative to positive by the over addition of positively charged flocculants. This overdosing mechanism is detailed in Figure 2.10 where wastewater, a biological colloid, is over dosed with ferric chloride leading to the resuspension of the colloid and the reversal of turbidity and COD removal. The use of zeta meter instruments gives excellent insight into targeting reduced colloid charges for optimum COD and biomass removal. Zeta potential measurements are favored over jar tests due to their quick and accurate results over the slow settling and inaccurate jar test measurements. The use of zeta potential measurements for characterizing and optimizing flocculation processes is discussed further in Section 2.6.

Several other technologies can also be employed with jar testers to enhance the process of optimizing flocculant dosing and velocity gradients. These technologies have been proven helpful but employ complicated and expensive instruments such as streaming current detectors, zeta potential meters, and particle sizing instruments (Adgar et al., 2005; Hall, 1965; Kim et al., 2008). The integration of these techniques is termed as “on-line” flocculation monitoring and has been well researched and commercialized for wastewater treatment processes.

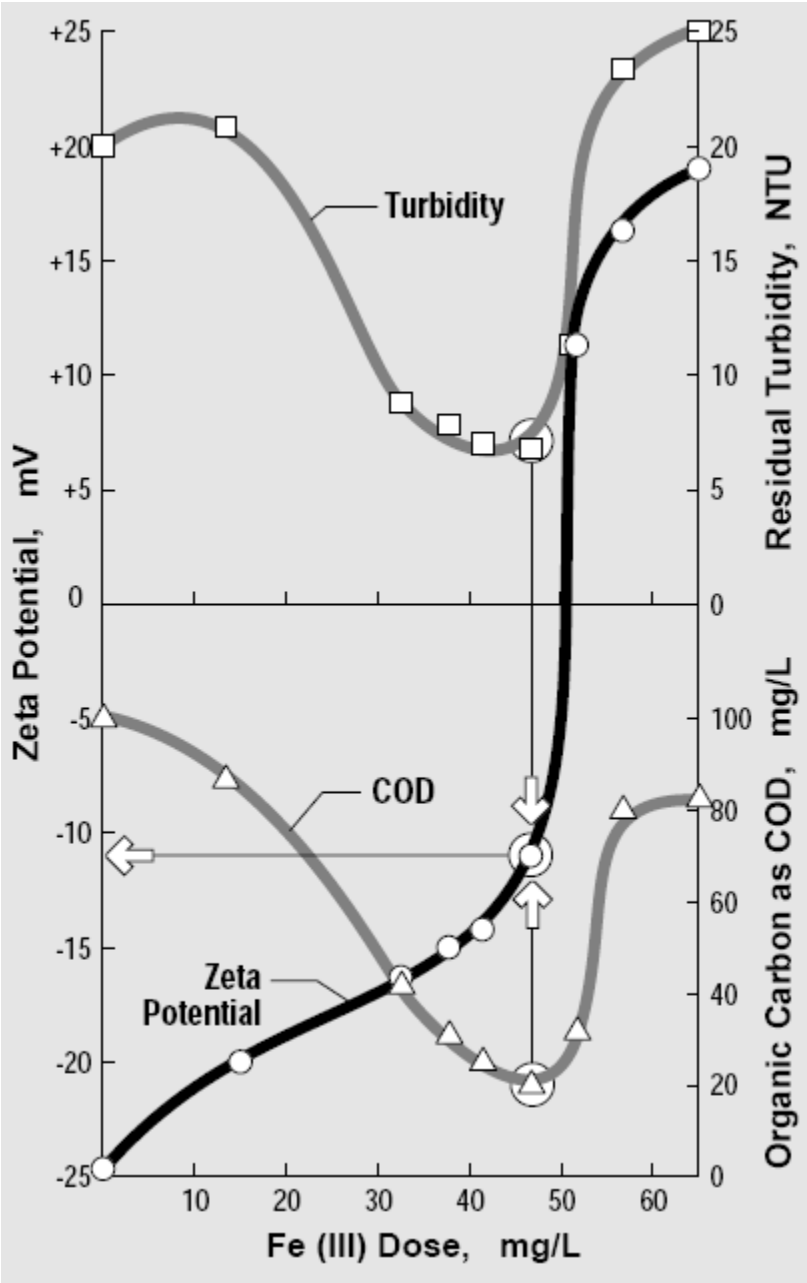


Figure 2.10. Charge neutralization and charge reversal with Ferric Chloride (Ravina, 1993).

2.5 Double Layer Compression and Surface Charge Reduction by Electrolyte Charge

Charged molecules and particles suspended in electrolyte solution are surrounded by "a cloud of ions of both signs" which forms the electric double layer (Harrison, 2003). The electric double layer consists of a fixed inner region of adsorbed counter ions and a diffuse region of negatively and positively charged hydrated ions (Figure 2.11). The thickness of diffuse layer is inversely proportional to the ionic strength of the solution, and thus, the addition of electrolytes reduces the radius of the double layer around charged particles allowing for cell-cell interaction and aggregation.

There are several different mechanisms of aggregation that can occur during flocculation and determining which mechanism governs the flocculation process is difficult. The most commonly used technique for characterizing the stability colloid suspensions is the measurement of particle zeta potential. The zeta potential (ζ) is a physical measurement of the electric potential at a particles shear plane (shown in the figure on p. 52), which corresponds to the net particle charge (Harrison, 2003).

The algae suspension is a colloid of negatively charged algae cells that can be destabilized by the addition of positive counter ions. Multi-valent counter ions such as Ca^{2+}, Mg^{2+}, Fe^{3+}, and Al^{3+} are more efficient at reducing the electric potential than mono-valent ions such as Na^{1+} because they have higher concentration of counter electric charge per atomic area. The level of charge reduction required to destabilize a colloid is different between algal species and can depend on the particles charge density, size, and shape, as well as extracellular organic matter (EOM) present in algal

suspensions (Henderson et al., 2008b). Algae suspensions usually require multi-valent counter ions for destabilization due to algae's strong negative charge, light weight, and steric repulsion caused by extracellular organic material (Bernhardt and Clasen, 1991).

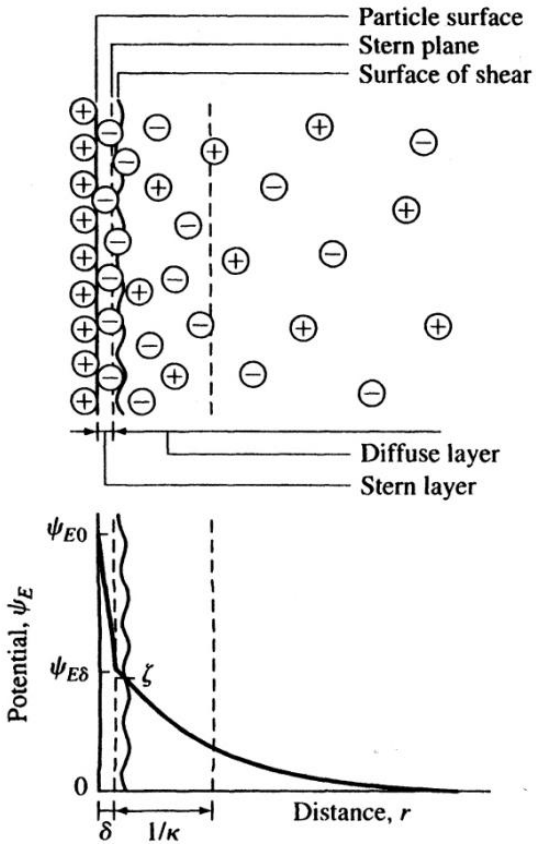


Figure 2.11. Representation of the ionic composition, electric potential (ψ), and zeta potential (ζ) at a colloid particle surface (Harrison, 2003)

2.6 Measuring and Characterizing Colloid Destabilization by Zeta Potential

Henderson (2008b) investigated the colloid destabilization of four algae species of different size, structure, and natural zeta potential by using zeta potential measurements. *Asterionella formosa*, *Melosira sp.*, *Microcystis aeruginosa*, and

Chlorella vulgaris were all flocculated with aluminum sulfate by Henderson (2008b). It was shown that a zeta potential reduction from -25 mV to around -10 mV was sufficient to destabilize and flocculate algae suspensions. The exact zeta potential values for optimal removal of the four species differed. For example, for , optima removal of *Chlorella vulgaris* the zeta potential had to be reduced to less than -16.8 mV, whereas *Asterionella formosa*, *Melosira sp.*, and *Microcystis aeruginosa* required zeta potential values less negative than -12.5 mV, -13 mV, and -15.5 mV, respectively.

In the same study (Henderson et al., 2008b), the amount of ionic flocculant required for optimal algae flocculation and removal was expressed in nanograms of aluminum per cell (ng/cell) (Figure 2.12). The study determined that 0.0028 and 0.0057 ng Al/cell were needed to neutralize *Microcystis aeruginosa* and *Chlorella vulgaris* cultures. The onset of flocculation started at lower dosage ratios, and almost 90% removal could be achieved at an Al to cell ratio of 0.001 ng Al/cell for *M. aeruginosa* and 0.002 ng Al/cell for *C. vulgaris* (Henderson et al., 2008b). The different flocculant dosage required for these two species reflects their significantly different surface charge density – 300 μeq/m^2 (*C. vulagris*) vs. 40 μeq/m^2 (*M. aeruginosa*).

This study demonstrates that 1.) charge reduction is important mechanism for flocculation by sedimentation or flotation and 2.) zeta potential measurements could be used for process control (Adgar et al., 2005). The algae species measured in Figure 2.12 displayed a Logarithmic relationship between charge neutralization (mV) and aluminum dosing (ng Al/cell). The species tested all showed charge reduction in the order of their cell charge density (neq/cell), with increasing cell charge density requiring more ng of

aluminum per cell. Determination of the charge density of other species could help determine the amount of Aluminum required for optimum charge reduction if measured under the same conditions of pH 7 and low ionic strength (Henderson et al., 2008b).

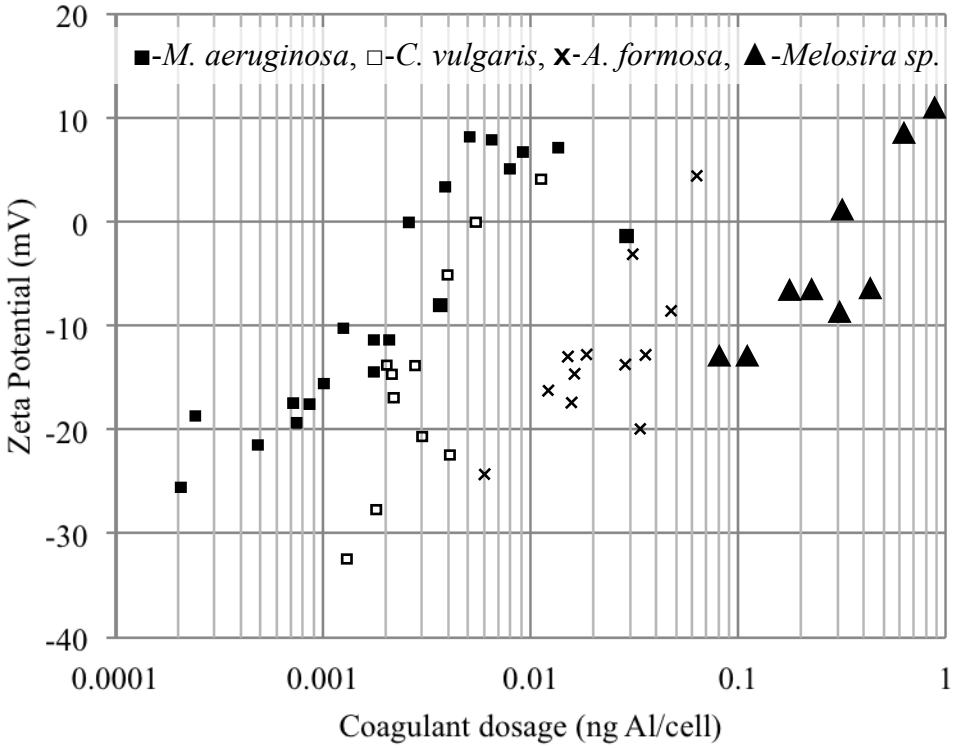


Figure 2.12 Effect of flocculant dosage on algae zeta potential at pH 6 (Henderson et al., 2008b).

3. MATERIALS AND METHODS

3.1 Culture and Growth Conditions

3.1.1 Strains

Nannochloris oculata (UTEX #: LB 1998) was acquired from Lou Brown at the Texas Agrilife Research Station in (Pecos, Texas).

Nannochloris salina 1776 was obtained from Los Alamos National Labs (Los Alamos, New Mexico), and given to us by Lou Brown from Texas Agrilife Research (Pecos, Texas).

3.1.2 Media Preparation

Nannochloris occulata was grown in modified Erdschreibers media (Table A.1.1). The modified Erdschreibers media consists of 15 g/L NaCl, 30 mg/L KH_2PO_4, which was modified from 10 mg/L KH_2PO_4, 400 mg/mL $NaNO_3$, which was increased from 100 mg/L $NaNO_3$, 34 mg/L H_3BO_4, 1 mL of trace metal stock solution (Appendix A.1.1.) and 0.5 ml of vitamin stock solution (A.1.). 15 g/L of$NaHCO_3$ was added to the media to serve as a CO_2 supply.

Nannochloris salina was grown in a modified F/2 media. The composition of F/2 media is listed in Table A.1.3 was acquired from UTEX. The modified media in

(Table A.1.4) contained 750 mg/L $NaNO_3$ instead of 75 mg/L. The vitamin, trace metal, and phosphate solution concentrations remain the same.

Individual trace metal stock solutions were prepared in volumetric flasks. Metal salts were dissolved in DI water. Each solution was mixed, autoclaved, and stored at 4 °C. To prepare the final trace metal stock solution, fixed amounts of individual trace metal stock solutions were mixed in a 1 L volumetric flask in a laminar fume hood. Trace metal stock solution was raised to 1 L volume, autoclaved, and stored at 4 °C.

Vitamin stock solution was prepared by adding vitamins to pre-autoclaved water inside a laminar fume hood. New vitamins and stock salt solutions, not including trace metals, were remade every two month to avoid contamination. All salts and vitamins were kept as sterile as possible and at 4°C to deter contamination.

The final media mixture for the algae was prepared by adding salts with sterile transfer pipettes, from the autoclaved stock solutions in a laminar fume hood, except for vitamin stock. All of the salts were put into stock solutions for accuracy and uniformity of prepared media. The media was autoclaved for 30 minutes (250°F for 30 minutes). Autoclaved media was immediately removed to avoid excess water evaporation and then allowed to cool at room temperature before adding vitamins inside the laminar fume hood with a sterile transfer pipette.

3.1.3 Inoculation Conditions

To inoculate new cultures, media was added to algae stock in a laminar flow hood. All media that was prepared under sterile conditions and kept at 4°C was warmed to room temperature before inoculating new cultures.

Algae was grown in Erlenmeyer flasks (500 mL to 4 L) with two-hole rubber stoppers, with one hole used to deliver air through a sterile transfer pipette for bubbling and the other hole used as a sampling port with another sterile transfer pipette with tubing attached for sampling semi sterile algae samples. A third hole was made for excess air to escape so the vessel does not pressurize. The air was delivered by an air pump (Aqua culture® pump, MK-1504), and filtered through a 0.2 micron acrodisc syringe filter (PALL #PN4612) fitted onto a sterile pipette. Air bubbling was done continuously to prevent algae settling, and provide excellent mixing and light exposure.

3.1.4 pH Adjustment

Culture pH was maintained around pH 9 by the addition of CO_2, which when dissolved in water forms carbonic acid and lowered the culture pH. Algae naturally raise their own pH daily to about 9.7 which will eventually slow its growth. CO_2 adjustment was done while monitoring the pH with a pH controller (Milwarkee, SMS122 pH meter), attached to a solenoid (JimBen Sun CO. Model A2A), which controlled CO_2 flow from a regulator on a CO_2 tank.

3.2 Monitoring Culture Conditions

Culture samples were taken from cultures through an inserted sampling tube. The sample was drawn into a sterile 5 mL syringe fitted at the end of the sampling tube. Sample pH was measured by a pH electrode (VWR #: SB90MS). The same electrode also measured dissolved oxygen and conductivity.

3.2.1 Optical Density

Algae density was determined by turbidity of the culture and measured by a spectrophotometer at 750 nm. For accurate turbidity measurements at 750 nm with linearly repeatable results, algae had to be diluted to between 0.25 to 0.75 OD (abs). A standard curve was constructed by taking a dense culture of OD 2.0 or more and measuring serial dilutions until the OD's were linearly correlated to their dilutions.

3.2.2 Dry Weight Measurements

Dry weights were used to determine the amount of biomass in the culture. There are two types of dry weights, a simple "dry weight" (DW), and an "ash-free dry weight" (AFDW). DW includes salts and inorganics in solution, which can be higher than 30 grams/liter in modified Erdschreibers media. Ash free dry weight (AFDW) gives the true amount of biological material grown in the culture without inorganic impurities. These weights were determined by weighing filters before and after the sampled algae have been filtered. Once filtered, the filter was dried to remove excess water and weighed to give the DW. To get the AFDW, the filter was put into a muffle furnace to remove

organic matter from the filter at 500 °C. The weight difference between the original dried filter, with algae and salts and the muffle furnace dried filter with only salts remaining gives the true ash free dry weight of the algae. Ash was the term that refers to salts and non-organic matter that make the dry weight higher than the true organic algae weight.

DW and AFDW SOPs are listed in Appendix 2. It is important to note that the outlined procedures must always use 40mL of algae. If the cultures density is above 3 g/L, only 20 mL of sample should be filtered.

3.2.3 Microscopy

An inverted digital microscope (Nikon Eclipse TS100) was used to examine the cultures health and growth. A magnification of 20X (Nikon 20X 10.40 PH 1 APL WD 3.1) was used to examine cultures, but 100X could be used to identify algae species for contamination identification.

Culture health was monitored by identifying possible contaminants such as bacteria or cyanobacteria. Bacteria are rod like shapes that are often connected to several other bacteria cells forming a filament. Filamentous bacteria can quickly overgrow single algae cells. The algae culture could develop a blue tint if blue-green cyanobacteria contamination occurs.

3.2.4 Counting Cells

Cell density expressed as the cells/mL of culture was measured by a hemocytometer (Brightline line hemocytometer, 15170-172, VWR). The hemacytometer is a specialty reusable glass slide with etched grids that form a viewable volume of 1 microliter in which to count cells. Counting cells using a hemocytometer is a tedious method with variable results. For better precision no more than 150 cells per 0.04 microliter should be aliquoted. At least 3 random squares were counted for each sample. If the three squares counted were drastically different it was suggested to count 3 more random squares, or to look for contamination.

Cell counts were done at least every other day to monitor culture health and growth phase. When constructing a growth curve, cell density measurements were taken every 12 hours.

3.3 Flocculation Process Measurements

3.3.1 Algae Flocculation

A solution of $1*10^7$ or $1*10^6$ cells/mL algae was prepared by diluting a denser culture with appropriate media. 50 mL of the culture was adjusted to a desired pH. Fifty mL cultures with initial cell concentrations of ($1*10^6$ and $1*10^7$ cells/mL) were adjusted to desired pH using 5M HCl. Separate 50 mL cultures were used for each pH. The 50 mL cultures were continuously stirred with a stir bar at 500 rpm throughout the

experiment. 0.3 mL of sample was taken to determine the starting optical density before any pH adjustment and flocculant addition.

The pH of the culture was adjusted drop-wise with HCl and allowed to stabilize within +/- 0.1 pH units from the target pH. Flocculant was then added drop-wise from a standard solution of 0.04 g/L $AlCL_3*6H_20$ in water, to a desired amount per cell during continuous mixing at 500 rpm. The culture was mixed with a stir bar for 5 more minutes at 500 rpm after flocculant addition and an additional 12 minutes at 60 rpm to encourage flock formation.

After 12 minutes of slow mixing the stir bar was stopped and the culture was gently poured into a graduated cylinder while tilting the graduated cylinder at a 45 degree angle towards the beaker to reduce the shear impacted on the flocks as they were transferred to the graduated cylinder. The flocks were allowed to settle in the graduated cylinder whose long uniform shape allows for more accurate settling rate and concentration factor measurements.

3.3.1.1 Measuring Optical Density During Flocculation

Optical density was taken from a fixed point of the 30 mL mark of the cylinder which was 7.4 cm from the bottom of the algae volume in the cylinder. The optical density was important to characterize the removal efficiency and the final concentration factor. Optical density was taken at 0, 3, 5, 10, 20, 30, and 60 min, and measured as before by taking 0.3 mL of culture from 7.4 cm above the bottom of the bottom of the graduated cylinder and diluting the sample 1:10 before measuring.

3.3.1.2 Settling Rate, Removal Efficiency, and Concentration Factor

Settling rate measurements were taken by measuring the distance of the settling algae front over time. Measurements of the settling front height were taken as often as possible. The settling rate was determined by the equation:

$$\text{Settling Rate} = \left(\frac{\text{Distance settled (cm)}}{\text{Time (min)}}\right) \quad [3\text{-}1]$$

The efficiency of coagulation was measured by comparing OD at a fixed point. Samples were taken from a fixed point at 7.4 cm above the bottom of the graduated cylinder. The efficiency of algae removal, also known as the Removal Efficiency (RE), was calculated by the equation below.

$$\text{Removal Efficiency (RE)} = \frac{\text{Supernatant OD after flocculation}}{\text{Original culture OD}} \quad [3\text{-}2]$$

The concentration factor (CF) was determined by using the algae settling distance, and the flocculation processes removal efficiency. Concentration factor was reported once settling has stopped, or a settling rate of 0.0 cm/min was recorded. This was the point when the distance settled was by aluminum charge neutralization and not from cell death or overnight settling from lack of culture mixing. The concentration factor was determined by taking a ratio of the algae height before flocculation over the height after flocculation, and multiplying it by the removal efficiency to account for any

unsettled algae. The concentration factor can be computed without the removal efficiency, but this is misleading with the inclusion of removal efficiency.

$$\text{Concentration Factor (CF)} = \frac{\text{Un-settled algae height}}{\text{Settled algae height}} * (\text{Removal Efficiency}) \quad [3\text{-}3]$$

3.4 Zeta Potential Measurements for Charge Neutralization Characterization

3.4.1 Zeta Potential Measurement Method

Zeta potential measurements used two different algae densities of $1*10^7$ and$1*10^6$ cells/mL to characterize charge neutralization of the culture. The manufacturer suggested that dilute cultures ($1*10^6$ cells/mL) be used so that the samples turbidity did not interfere with calculating the colloids zeta potential. Additional steps were required for testing the dense samples ($1*10^7$) zeta potential. Dilutions were prepared by diluting a denser culture with the appropriate media. 50 mL was taken from the diluted algae and pH adjusted to the desired pH. A separate 50 mL culture was used for each pH and zeta potential measurement. The 50 mL culture was continuously stirred with a stir bar at 500 rpm. A separate beaker of the appropriate media was stirred at low speed to be pH adjusted to the same pH as the algae sample, and used to dilute samples to $1*10^6$ cells/mL before measuring zeta potential if taken from the dense $1*10^7$ cells/mL culture.

A sample of 1 mL was taken to test the starting zeta potential before any pH alteration or flocculant addition. If the sample was taken from the dense culture ($1*10^7$)

it was diluted to the manufacturers suggestion by taking 0.2 mL of algae from 1 cm below the meniscus and adding it to 1.8 mL of the appropriate media that has been pH adjusted to the same pH as the dense sample that was being taken for a dilution of 1:10 to $1*10^6$ cells/mL. The diluted sample from the dense culture was then vortexted at full intensity for 10 seconds to ensure that all flocks have been broken so that they will not interfere with zeta potential measurements. The pH levels of the dilution media and sample were within +/- 0.1 pH units to the target pH otherwise the dilution will alter the algae's environment and the ionic species that were present in the sample to be tested. After testing the dense cultures diluted sample, 1 mL of dense algae was taken from the 50 mL culture, vortexted for 10 seconds at full intensity, and tested for its zeta potential without being diluted to determine if dilution of the dense culture samples was necessary. The comparison of these measurements is detailed in Appendix 3.

The pH of the culture was then adjusted drop wise while stirring with a stir bar at 500 rpm with 5M HCl to the target pH, and allowed to stabilize within +/- 0.1 pH units from the target pH. The media used to dilute the dense samples before testing was also pH adjusted to the same pH. Another sample was taken, diluted if from the dense culture, and vortexted before testing the zeta potential of the pH shift with no flocculant. If testing the dense culture a sample of undiluted dense culture was also tested as before. The flocculant was then added drop-wise from a standard solution of 0.04 g/L $AlCl_3*6H_20$ (Alfa Aesar, stock #: 12297), to a desired concentration with continuous mixing at 500 rpm. The desired concentration was calculated in nanograms of flocculant per cell to but still expressed at g/L. Two minutes after adding the flocculant, a sample

was taken from 1 cm below the meniscus, and diluted if necessary with media at the same pH to $1*10^6$ cells/mL. The sample was then vortexted and its zeta potential measured. Ten minutes after flocculant addition the measuring process was repeated as before because the zeta potential has been reported that zeta potential can take up to 7 min to stabilize (Clasen et al., 2000). A sample of dense undiluted culture (1E7 cells/mL) was also tested after the dilute sample at 10 minutes was sampled and tested.

The Zetasizer NanoZS instrument took at most 100 individual zeta potential measurements of the culture sample to be averaged out to a single zeta potential measurement. The instrument would keep taking measurements until the results were within an acceptable range of variance, but would stop at 100 measurements. This was repeated three times to achieve a standard deviation of the accuracy of the measurements. Each of the three measurements took between 30 sec and 2 minutes depending on the number of individual measurements taken to be averaged out to a single zeta potential measurement. Zeta potential measurements by the Malvern Zetasizer nano were taken with the following specification in the programs settings menu. The material of interest was protein due to the algaes size and insoluble form. The Refractive Index (RI) = 1.427, and an absorbance of (OD)=0.00 abs was used. The altering of these settings did not change the zeta potential readings, and was advised against by the manufacturer.

3.4.2 Ionic Conductivity Manipulation for Coagulation Experiments

Understanding how salt water and high conductivity effects coagulation requires the manipulation of the ionic content of the media. To change the ionic content of algae for coagulation, the cells were removed from their suspension by centrifugation at and re-suspended in new media with a different ionic content. Care was taken to ensure that resuspended cells did not form flocks by viewing the culture under a microscope. If necessary the culture was diluted to the correct density with DI water before centrifugation. Then the culture was centrifuged at 3000 g for 5 minutes and the supernatant was very carefully decanted to make sure that no algae cells were lost. The amount of removed supernatant was then replaced with the same amount of new buffer of known ionic content to ensure that the cell density didn't change.

If the culture was not as dense as the culture of interested then the removed supernatant was replaced with a lesser amount of the buffer media of interest to increase the cell density. Additional media washings by centrifugation and decanting will replace the removed supernatant with the same amount and type of media of known ionic content. This process was repeated until the conductivity was similar to that of the new media of interest. The culture was then coagulated as explained above.

4. RESULTS AND DISCUSSION

Flocculation is an important process for efficient and cost effective harvesting that can be brought about by the addition of multi-valent ionic species such as Al^{3+}, which can reduce repulsive forces between algae cells and initiate flocculation. The mechanism of charge reduction and subsequent flocculation is sensitive to pH, ionic content, and flocculant dosage.

4.1 pH Effect on Flocculation Efficiency at Different $AlCl_3$ Concentrations

To determine if pH adjustment would be beneficial to ionic flocculation with $AlCl_3$, settling (Figure 4.1) and removal efficiencies of flocculated algae (Figure 4.2) were evaluated at three different pH's and $AlCl_3$ concentrations. The settling progress was monitored by measuring the movement of the algae suspension interface over a period of more than 80 minutes (Figure 4.1). The settling of the algae front in cultures at pH 7 and 9 could not be clearly identified because of the processes poor removal efficiency at pH 7 and 9. Therefore the settling measurements began when the algae front could be clearly seen shortly after 10 minutes when the algae began to concentrate from settling. The fastest settling occurred at pH 5 and 0.55 g/L aluminum chloride (Figure 4.1.c). After 10 minutes, the algae interface came to rest (no further change in distance) indicating that the sedimentation process was complete. The average sedimentation rate with 0.55 g/L $AlCl_3$ at pH 5 was approximately 2 cm/min, taking less than 5 min to settle. At pH 7 and 9, settling was slower stopping after 20 and 30 min

respectively. Estimated sedimentation rates were 0.5 cm/min for pH 7 and 0.3 cm/min for pH 9. Further analysis of Figure 4.1 shows that higher $AlCl_3$ dosages of 1.8 g/L and 2.7 g/L $AlCl_3$ reduced the initial and average settling rates. These results suggest that higher $AlCl_3$ dosages and higher pH hinder the algae settling rate and distance. The reduction in settling rates can be explained by the possible formation of insoluble aluminum hydroxide species ($Al_x(OH)_y$) at pH 7 and 9 as shown in Figure 2.4. The slowest settling was observed at pH 9 followed by pH 7 and 5 (Figure 4.1). At pH 9 and 2.8 g/L $AlCl_3$, it took more than 90 minutes for the interface to stop moving.

(a)

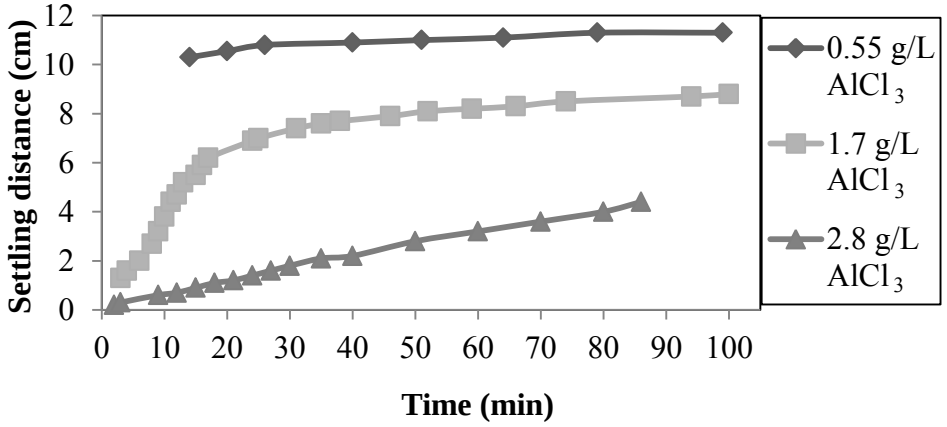
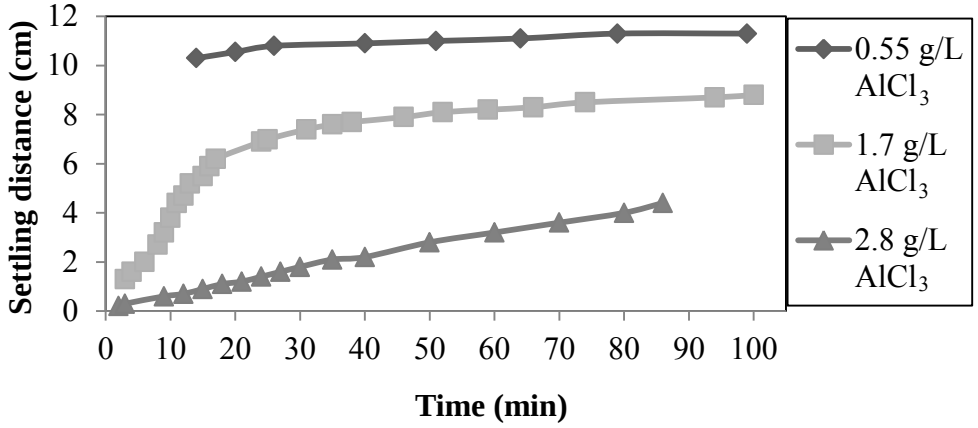


Figure 4.1. Distance of *N. occulata* settling after flocculation with $AlCl_3$, (a) pH 9, (b) pH 7, (c) pH 5.

(b)



(c)

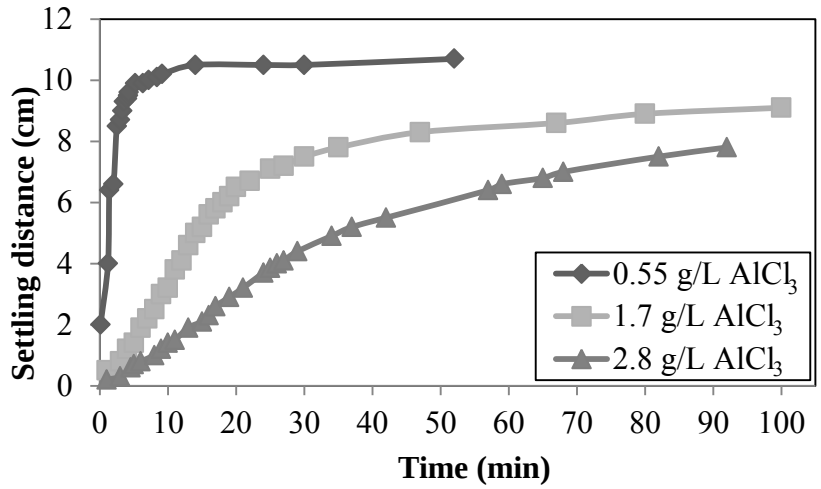


Figure 4.1 Continued.

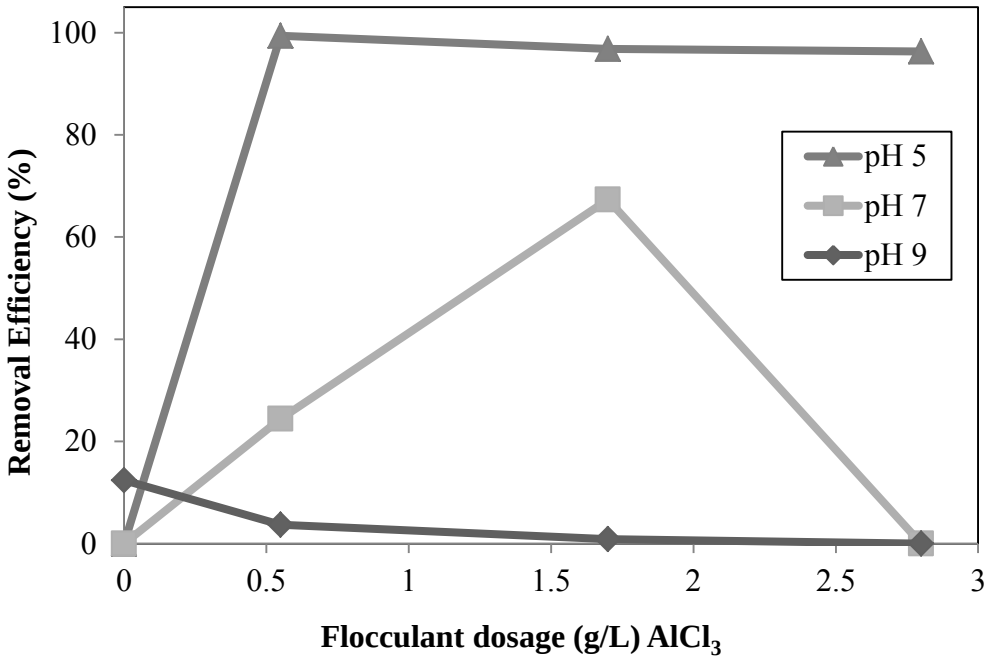


Figure 4.2. Flocculation efficiency vs. $AlCl_3$ dosage (g/L) as a function of dosage and pH.

Neither pH nor $AlCl_3$ dosage had an effect on cell removal at pH 9 (Figure 4.2). A slightly acidified culture (pH 7.0) had greater removal efficiencies of 24.4% and 67.3% at flocculant dosages of 0.5 g/L and 1.7 g/L, respectively. This increase in removal efficiency is probably the result of charge reduction by the flocculant rather than pH adjustment because culture acidification to pH 7 without $AlCl_3$ did not initiate flocculation (Figure 4.3). The apparent detrimental effect of 2.8 g/L $AlCl_3$ at pH 7 (Figure 4.2) was the result of an erroneous turbidity measurement. The turbidity in these experiments was measured at 3.4 cm below the meniscus of the measuring cylinder. As shown in Figure 4.1b, after 60 minutes at 2.8 g/L $AlCl_3$ and pH 7 the front of the settling algae had not passed the sampling point at 3.4 cm, and turbidity of the settling

suspension was measured rather than that of the clarified supernatant. Nevertheless, pH 7 displayed a vaguely linear relationship between flocculant dosage and algae removal efficiency from 0 g/L to 1.7 g/L $AlCl_3$. Flocculation at pH 5 resulted in a significant increase in removal efficiency compared to pH 7 and 9. At pH 5 flocculation removal efficiencies with the three flocculant concentrations were all above 95%. The greatest difference between pH 5 and 7 was observed at 0.55 g/L $AlCl_3$. At this dosage, the flocculation at pH 5 resulted in 98% removal efficiency compared to 24.4% at pH 7. The increase in removal efficiency at pH 5 compared to the other two investigated pHs is consistent with previous that used *Chlorella sp.* and determined the optimal flocculation pH range for this green algae was between 5 and 6 (Bernhardt and Clasen, 1991; McGarry, 1970).

4.2 Charge Neutralization by Acid Titration on Algae Zeta Potential

To help explain the observed pH effect on flocculation, the zeta potential of *N. oculata* was measured as a function of pH ranging from 1.5 to 11 (Figure 4.3). The zeta potential of the algae did not change significantly by titration from pH 11 to pH 5.5. A measurable charge reduction of *N. oculata* by HCl titration was observed below pH 5. The reduction in the zeta potential from -25 to -20 mV was first observed at pH 4.5, with further reduction to -11.9 mV at pH 1.7.

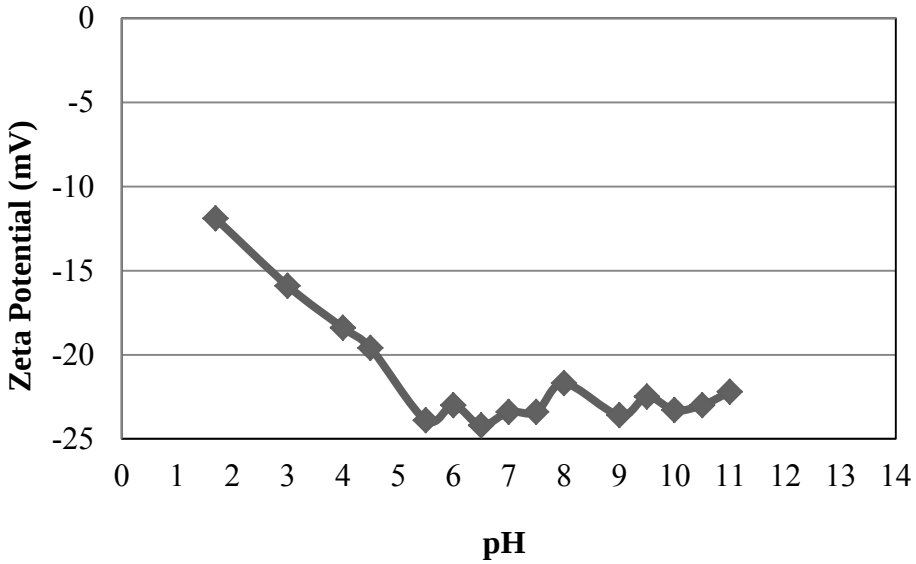


Figure 4.3. Effect of pH on zeta potential of *N. oculata* culture acidified with HCl.

The pH region of zeta potential change coincides with the behavior of carboxylic acid residues, which have an isoelectric point of 4.3. The cell wall and organic material that makes up the extracellular matrix contain carboxyl groups, which give the cell its charge, and could possibly explain the change in zeta potential at pH 4.5 (Figure 4.3) which corresponds with carboxylic acids relative isoelectric point (Henderson et al., 2008a; Jensen, 1984).

4.3 pH Effect on Zeta Potential With and Without $AlCl_3$ Flocculant

Although charge reduction is one of the prerequisites for initiating cell aggregation, acid titration alone did not readily initiate algal flocculation. Based on data shown in Figure 4.2, it appears that an increase in flocculation efficiency and recovery of algal biomass could be achieved by a combined charge reduction produced by positively

charged $AlCl_3$ species and pH adjustment by acidification. The synergistic effect of pH and flocculant on the zeta potential of *N. oculata* suspension was investigated and plotted in Figure 4.4. The addition of 0.5 g/L $AlCl_3$ to acidified algae suspensions of $1*10^6$ cells/mL, resulted in a neutralization of negative zeta potential between pH 7.5 and 4.5. Full charge neutralization at 0 mV occurred at pH 5.3, whereas charge reversal was seen at pH 4.5. The positive zeta potential of +10mV at pH 4.5 was due to an over dosage of positive aluminum ions. Charge reversal by over dosing can cause a re-stabilization of algal colloid by creating positive repulsive forces between cells with positively charged aluminum species adsorbing to the algae surface (Duan and Gregory, 2003). Charge neutralization and reversal was not caused by acidification as shown by the control tests with no $AlCl_3$. The algae control without flocculant maintained a constant zeta potential despite acidification to below pH 5.

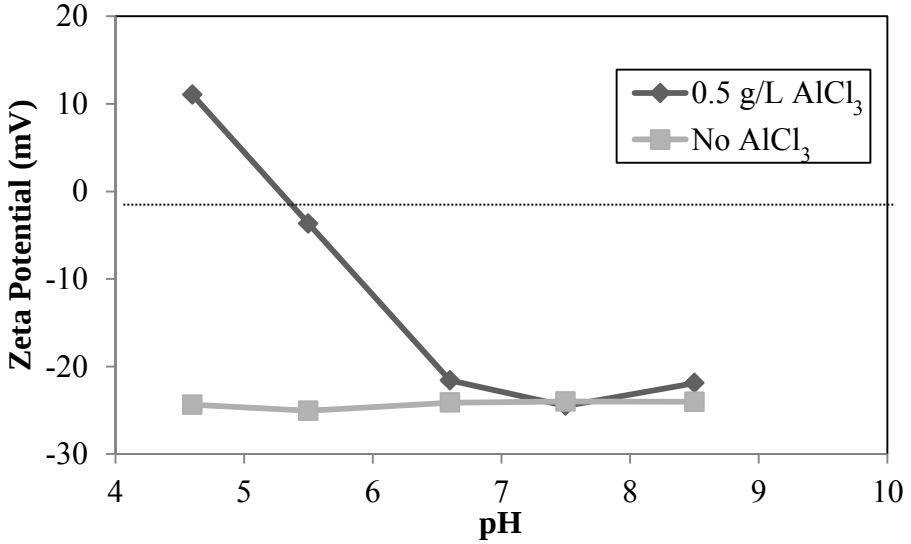


Figure 4.4. Effect of pH on zeta potential of *N. oculata* at $1*10^6$ cell/mL with and without 0.5 g/L $AlCl_3$ (g/L).

It can be concluded from the data presented in Figure 4.4 that the addition of $AlCl_3$ was the primary cause for the algal charge reduction in the acidic pH region. This experiment identified pH 5.3 as the point of charge neutralization with 0.5 g/L $AlCl_3$, thus, pH 5.3 was used in subsequent *N. oculata* flocculation studies.

4.4 Reduction of $AlCl_3$ Dosage at pH 5.3

To determine whether flocculant dosages lower than 0.5 g/L could initiate algae flocculation, a range of $AlCl_3$ concentration from 0.025g/L to 0.55 g/L were tested at pH 5.3 with *N. oculata* concentration of $1*10^7$ cells/ml. The removal efficiencies in Figure 4.5 indicated that greater than 95% algae recovery can be achieved with flocculant concentrations from 0.05 g/L to 0.55 g/L. Based on this data it appears that a flocculant dosage of 0.05 g/L at pH 5.3 would be sufficient for 95% recovery of *N. oculata*. This relatively low dosage makes $AlCl_3$-mediated flocculation process potentially practical because flocculant price is one of the cost drivers of algae harvesting. In addition, the lowest possible dosage of toxic metallic flocculant will not only reduce processing cost, but will make it easier to develop viable water recycling options and co-products from lipid-free algal biomass.

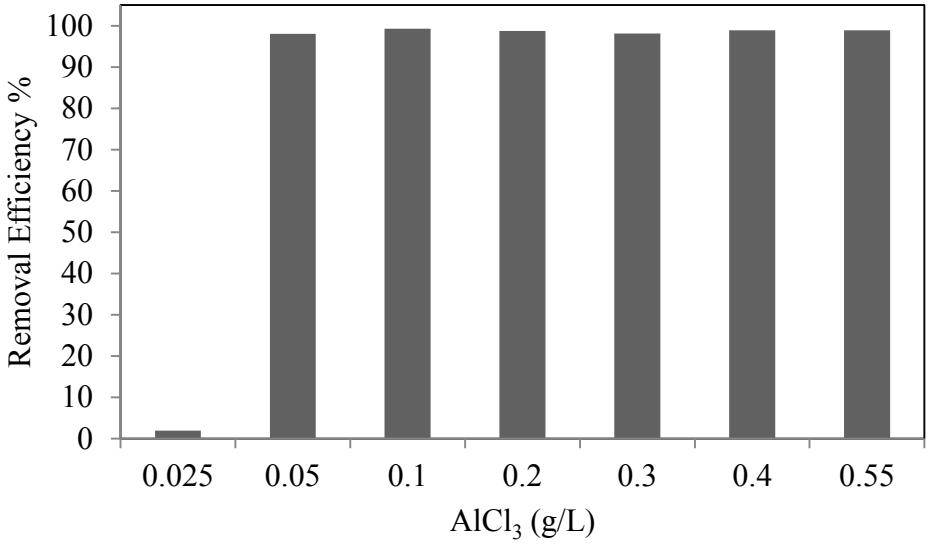


Figure 4.5. The effect of flocculant dosage on removal efficiency with *N. oculata* algae concentration of 10^7 cells/ml at pH 5.3.

4.5 Effect of $AlCl_3$ Dosage on Concentration Factor

Another measure of flocculation efficiency is the achievable concentration factor. The concentration factor (CF) is used as a representative value of the amount of water removal during sedimentation of flocculated algae (Lee et al., 2009). The concentration factor includes both the distance traveled by the settling algae supernatant interface and removal efficiency, and is estimated by equation [3-3].

$$\text{ConcentrationFactor (CF)} = \frac{\text{Unsettled Algae Height}}{\text{Settled Algae Height}} * (\text{Removal Efficiency}) \quad [3\text{-}3]$$

Figure 4.6 shows that the decrease in concentration factor with the increase in $AlCl_3$ flocculant dosage at pH 5.3 and 5. Increase in flocculant dosage interfered with

the settling rate and distance of algae flocks, as seen in Figure 4.1. $AlCl_3$ concentration of 0.05 g/L $AlCl_3$ resulted in the highest concentration factor and subsequently the highest % TSS in the settled algae, while 0.025 g/L dosage did not initiate algae aggregation and flocculation. Although the removal efficiencies with flocculant concentrations above 0.05 g/L were all greater than 95%, concentration factors were significantly deficient with increased $AlCl_3$ concentration. For example, $AlCl_3$ dosages of 0.05 g/L and 0.1 g/L resulted in near complete algae removal (> 95 %) but the concentration factors were19.6 and 14.2, respectively. The concentration factor of 19.6 at pH 5.3 using 0.05 g/L $AlCl_3$ resulted in a 97% water removal, thus, achieving the desirable goal for algae harvesting, i.e. high concentration factor and removal efficiency using low flocculant concentration. This example demonstrates the usefulness of concentration factor as a performance criterion for algae flocculation. The decrease in concentration factor is consistent with the observed decrease of sedimentation distance over time with increasing the $AlCl_3$ dosage (Figure 4.1). The formation of $Al(OH)_3$ precipitates at higher $AlCl_3$ dosages could explain both the reduced sedimentation rates and lower concentration factors.

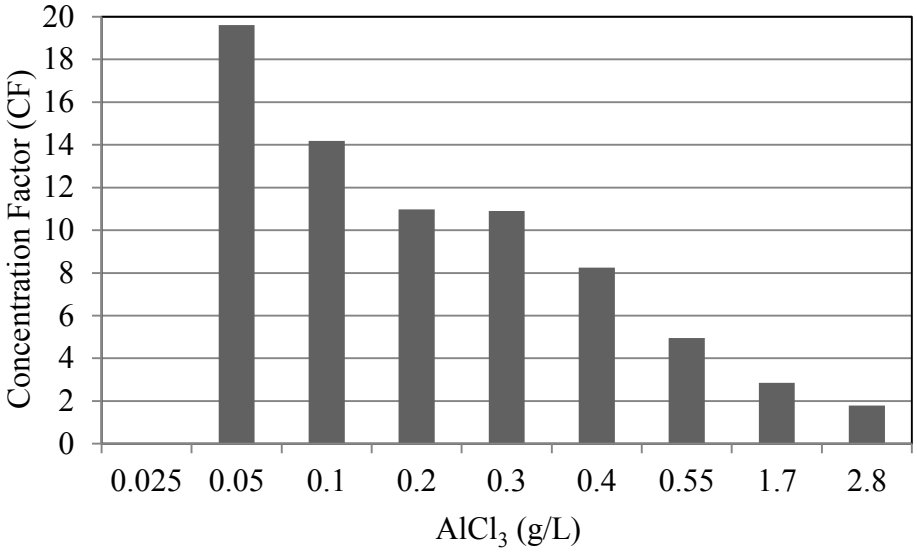


Figure 4.6.The effect of flocculant dosage with *N. oculata* algae concetration of 10^7 cells/ml at pH 5.3.
*Note: 1.7 and 2.8 g/L dosings were at pH 5.0, instead of pH 5.3.

4.6 Effect of $AlCl_3$ Dosage and NaCl Concentration on Zeta Potential and Colloid Destabilization

4.6.1 Salt (Ionic Strength) Effect on Zeta Potential With and Without Flocculant

As discussed in Section 2.5, the ionic strength is inversely proportional to the thickness of the electric double layer around colloid particles and reduces the amount of energy needed to cause cell-cell attraction and aggregation with the increase of ionic strength. Since the modified Erdschreibers media used for growing *N. oculata* contains 15 g/L NaCl, we wanted to determine the effect of NaCl on *N. oculata* flocculation.

The effect of NaCl concentration on measured zeta potential of *N. oculata* culture with and without 0.1 g/L $AlCl_3$ is shown in Figure 4.7. To control the ionic strength and

eliminate the effect of evaporation on salt concentration, algae cells were centrifuged and re-suspended in fresh media containing 0, 7.5, and 15 g/L NaCl. The zeta potential measurements of the control culture (0 g/L $AlCl_3$) show a linear reduction of the zeta potential with increasing salt content from 0 to 15 (g/L). The addition of 0.1 g/L $AlCl_3$, as expected, further reduced the zeta potential while maintaining the linear correlation between the zeta potential and NaCl concentration. The charge reduction achieved by the NaCl and $AlCl_3$ ions points to their synergistic activity and the potential benefit of NaCl in the formulated media.

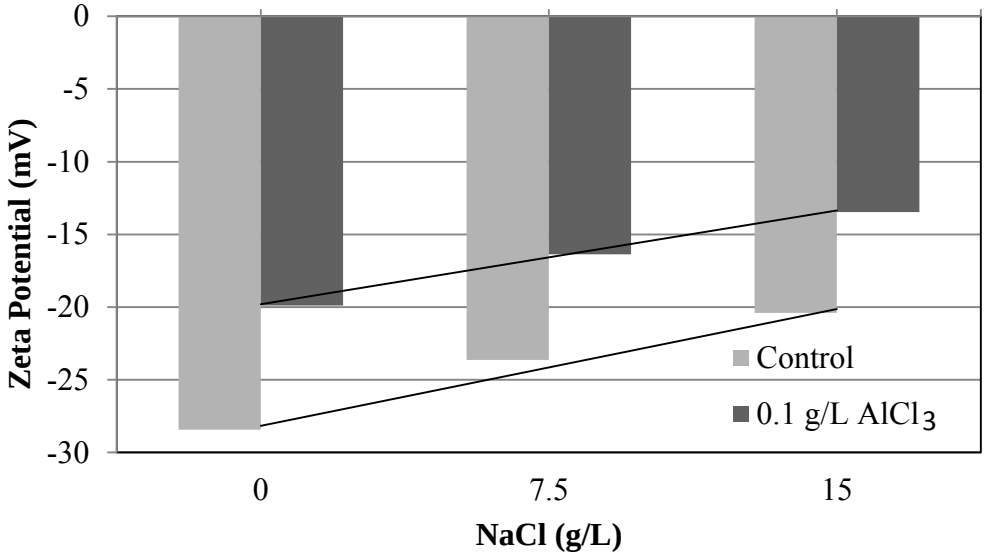


Figure 4.7. Effect of NaCl concentration on charge neutralization with 0.1 g/L AlCl3 at pH 5.3 with 1*106 cells/mL

4.6.2 Effect of $AlCl_3$ Dosage to Cell Ratio on Zeta Potential and Destabilization

Flocculant dosages of 0.1 g/L and 0.5 g/L $AlCl_3$with $3.07*10^7$ cells/mL correspond to dosage ratios of 0.000662 and 0.00331 ng Al/cell, respectively. The comparison of the charge reduction by these dosages expressed in (ng/cell) level enables the comparison to similar algae charge neutralization studies of *M. aeruginosa* and *C. vulgaris* species with aluminum salts by Henderson et al., (2008b). Despite differences in methodology by Henderson et al., (2008b), i.e. freshwater species in low salt buffer and flocculation at pH 7 with Al_2SO_4, dosing of 0.000661578 and 0.003308297 ng Al/cell in this study behaved similarly to cause charge reduction of *N. oculata* at pH 5 and 6 with $AlCl_3$. The dosing of $AlCl_3$ at pH 5 neutralized *N. oculata*'s charge remarkably similar to, and slightly more than, how Al_2SO_4 reduced *M. aeruginosa*'s charge at pH 7. This gives excellent evidence to the difference between the optimum pH range of $AlCl_3$, identified in this study as pH 5.3, and Al_2SO_4, which was reported as pH 6.5 by Amos Richmond (1986). Al_2SO_4 is known to flocculate at higher pH levels (than $AlCl_3$) in part because SO_4^{2-} is known coagulant promoter with sulfates higher -2 charge (than Cl^-) acting as a precipitant forming complex aluminum sulfate species capable of flocculating (Shin et al., 2008). $AlCl_3$ however flocculates at lower pH levels because chlorines lower -1 charge does not act as a coagulant promoter (Shin et al., 2008).

Despite the lack of more than two data points in Figure 4.8, the difference in slopes between pH 5 and 6 helps explain the difference in ionic species valence, or charge density. As discussed in Section 2.3.3.1, ionic species with a higher valence charge (z) can flocculate algae at lower concentrations because of their increased counter

charge density. Since Figure 4.4 showed an increase in zeta potential, or charge reduction, from pH 6 to 5 with the same $AlCl_3$ concentration, it could be assumed that a higher charged ionic species existed at pH 5 than at pH 6. The dosing of 0.003308297 ng Al/cell with higher charged aluminum species at pH 5, neutralized *N. oculata* by 11.56 mV more than by the same aluminum concentration at pH 6. The difference between pH 5 and 6 grew to 19.26 mV with the addition of 0.000661578 ng Al/cell since the denser aluminum counter charged species at pH 5 contained more cationic charge per aluminum specie than the lower charge density aluminum species at pH 6. This denser collection of cationic charge per aluminum specie yielded the steeper curve at pH 5 over pH 6.

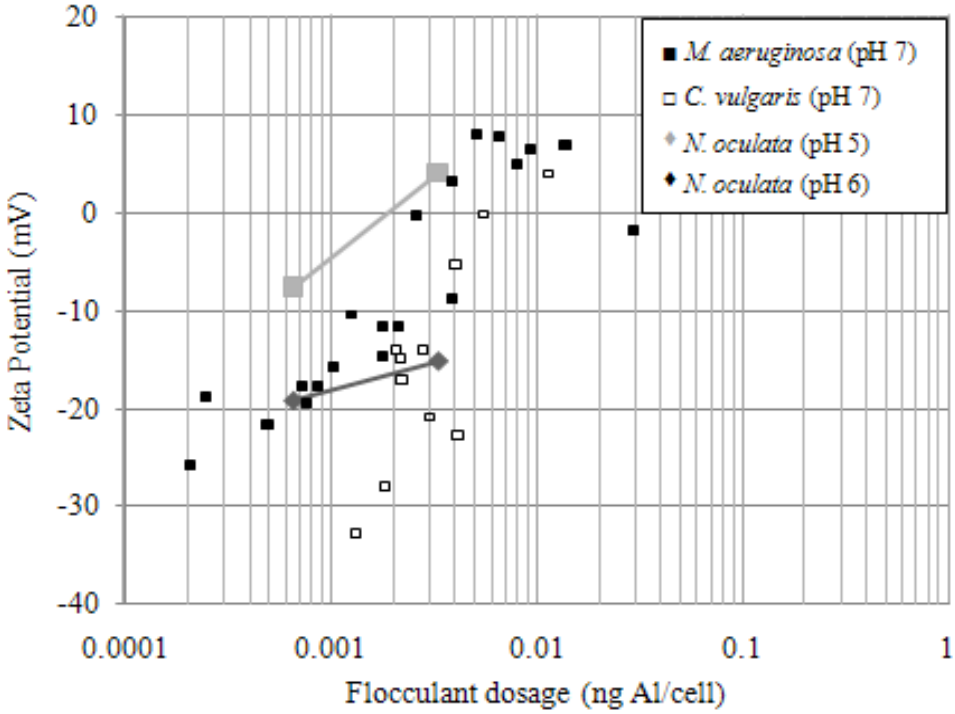


Figure 4.8. Charge neutralization vs. flocculant dosage (ng Al/cell) at pH 5 and 6.

4.6.3 Additional Look at the Effect of pH on *N. oculata* Destabilization and Flocculation: Observations and Speculations

The zeta potential measurements in the acidic region were expanded to determine the effect of further acidification on cell charge neutralization with $AlCl_3$ flocculant. Figure 4.9 displays the large change in zeta potential due to charge neutralization between pH 7 and 5 when using 0.5 g/L $AlCl_3$ flocculant. Below pH 5, there was a large increase in the negative zeta potential from +5 mV at pH 5 to -25 mV at pH 4 and then a slight increase to -20 mV at pH 3. This large reduction in zeta potential at pH 4 and small increase at pH 3 could possibly be explained by the concentration shift of charged aluminum hydroxide species due to additional acidification as shown in Figure 2.4 and 2.5. For example, acidification of $Al_8(OH)_{20}^{4+}$ species would case conversion to $AlOH^{2+}$ and then to Al^{3+} with additional pH reduction (Figure. 2.4). Unlike the flocculation experiments and zeta potential measurements performed by Henderson (2008a), we did not observe any significant flocculation using $1*10^6$ cells/mL cultures. Despite the linear relationship between pH and zeta potential and the absence of overall charge reduction at pH 3 and 4, the experiments with 0.1 g/L $AlCl_3$ dosage showed similar results to those with 0.5 g/L.

The lack of charge reduction and destabilization of *N. oculata* cultures at pHs below pH 5, could be possibly explained by altered the charge density of hydrolyzed $AlCl_3$ species to lower charged species. Figure 2.5 shows the hydrolysis of aluminum hydroxide species just above pH 4.5, which is closest pH (pH 4) where flocculation inhibition by acidification was observed. The interference is explained by the absence of

higher valence aluminum hydroxide species seen at pH 5 but hydrolyzed to lower valence species at pH 4 (Figure 2.5).

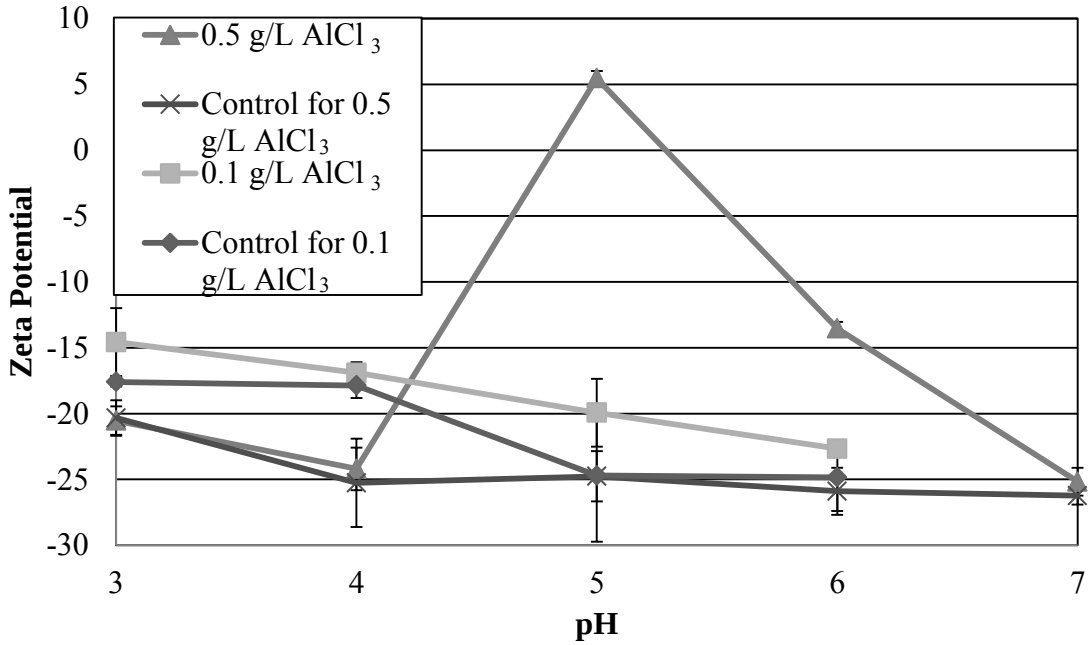


Figure 4.9.Effect of pH on zeta potential with and without 0.5 g/L $AlCl_3$ and 0.1 g/L $AlCl_3$.
*(The bars represent the standard deviation of 3 sample points averaged to produce the displayed data points)

The interference at pH 4 is also seen with quartz particles in Figure 2.8, as the electrophoretic mobility of the quartz particles was reduced from 1.5 to 0 ((μm/sec)/(V/cm)) when the pH was reduced from pH 5 to 4. This decrease in electrophoretic mobility of the quartz particles can also be attributed to a decrease in the positive cationic charge of the flocculating aluminum species. The pH interference seen in Figure 4.9 is similar to that seen in Figures 2.7 with charge reduction of wastewater

particles as well as in Figure 2.8 with flocculation of inorganic quartz particles (Bernhardt and Clasen, 1991).

The presence of charged aluminum species, Al^{3+} and $Al(OH)^{2+}$, at pH 4 is evident by electrophoretic mobility reduction of inorganic quartz particles at pH 4 (Figure 2.8). Since lower valence species such as Al^{3+} have been shown to neutralize particle charge at pH 4, an assumption was made that 0.1 g/L $AlCl_3$ was too low of a concentration to neutralize algae cell charge to cause colloid destabilization. This is also supported by the control experiment with 0.1 g/L $AlCl_3$ in Figure 4.9, which showed no interference by the algae on aluminum ions' ability to reduce the cell charge at pH 3 and 4.

Insufficient dosing level is the most likely explanation for the absence of charge reductions at pH 4 and 3. The relationship previously shown in equation [2-1] between aluminum concentration ($(C_i)_{flocc}$) and the charge of the multi-valent aluminum species (z) responsible for charge neutralization, supports the previous assumption that the aluminum concentration required for sufficient charge neutralization at pH 5 is too low for a similar charge neutralization with lower valence aluminum species at pH 3 and 4. To determine the amount of $AlCl_3$ required for charge neutralization, equation [2-1] can be used assuming poly-aluminum species of +4 valence at pH 5, +3 valence at pH 4, and +2 valence at pH 3. The aluminum solubility curve in Figure 2.5 does not show +4 valence species, but studies have detected the presence of $Al_7OH_{17}^{+4}$ and assumed their participation in the charge reduction process (Ravina, 1993).

The lowest effective dosage tested for efficient flocculation in this work was 0.05 g/L at pH 5.3. Using equation [2-1], which calculates the amount of salt required for

coagulation and assuming that the aluminum hydroxide species at pH 5.3 are all $Al_8(OH)_{20}^{4+}$, then 0.55 g/L of $AlCl_3$ would be required at pH 4 to cause coagulation with pure Al^{3+} species. Similarly, 64 times more $AlCl_3$ at pH 3 would be required if $AlCl_3$ hydrolyzed to a 2+ valence aluminum species such as $Al(OH)^{2+}$. There is likely a mixture of aluminum hydroxide species whose combination of positive valences of 4+ and higher allow for a significantly lower coagulation dosage at pH 5.3 than that required with 2+ and 3+ valence species at pH 3 and 4. In addition, aluminum hydroxide has a very low solubility which will increase the likelihood of aluminum hydroxide aggregate formation. The bridging of algae cells by cationic aluminum aggregates could drastically reduce the aluminum concentration required for flocculation (Xiaoying et al., 2009). Should polymeric like flocculation mechanism occur at pH 5.3, then $AlCl_3$ concentration required for flocculation at pH 4 and 3 could be significantly higher than those concentrations listed above, since equation [2-1] only accounted for charge neutralization and not polymer bridging.

5. CONCLUSIONS AND SUGGESTED FUTURE WORK

In conclusion, 0.05 g/L $AlCl_3$ dosage at pH 5.3 was the lowest effective flocculant dosage for flocculation of *N. oculata*. This dosage (0.05 g/L $AlCl_3$) yielded the maximum observed removal efficiency and concentration factor, making it the most effective. Higher aluminum flocculant concentrations hindered removal efficiency and concentration factor, requiring careful dosing practices to minimize the dosing of toxic metallic flocculants. As measured by algae zeta potential, a pattern of increasing charge neutralization by aluminum chloride occurred from pH 7 to pH 5. Charge neutralization did not occur at pH 3 and 4 with the 0.5 g/L flocculant due to the hydrolysis of highly charge aluminum hydroxide cations to the lesser charged aqua-metallic ions such as Al^{3+} and $Al(OH)^{2+}$ (Letterman, 1999; Shelef et al., 1984). It has also been hypothesized that the absorption of aluminum species to the cell wall can render aluminums positive charge ineffective at reducing the cells negative charge at the stern layer (Letterman, 1999; Shelef et al., 1984).

The possible mechanisms involved in *N. oculata* flocculation include double layer compression by NaCl in the algae media, charge neutralization by positively charged aluminum species such as $Al_8(OH)_{20}^{4+}$, $Al_6(OH)_{15}^{3+}$, and $Al(OH)^{2+}$, and possibly cell bridging by densely charged aluminum hydroxide species, such as $Al_{13}O_4(OH)_{24}^{7+}$. Reported success of particle flocculation by polyaluminum chloride species (PACl) at near neutral pH levels, challenges the idea that flocculation by monomeric trivalent ionic species is ideal.

Additional research must be done on aluminum flocculation and other concentration and harvesting techniques. Determining the aluminum species responsible for flocculation can help improve the process and could be determined by NMR, small angle X-ray techniques, and potentiometric titration techniques. Also, determining conditions for stable ionic flocculant species to exist without being subjected to hydrolysis could greatly increase the flocculants efficiency. The use of poly-nuclear ionic flocculants that can flocculate algae at a higher pH than 5.3 would reduce the cost of acidification, which could be a rather high cost item for media with a high buffering capacity. Efficient flocculation can be performed at higher pH levels than 5.3 by highly charged ionic hydroxides, which can be made with nontoxic ions such as calcium, or with the precipitation of cations (Ca^{2+}, Mg^{2+}, Al^{3+}, Fe^{3+}) by anionic coagulant promoters (SO_4^{2-}, PO_4^{3-}).

For example, calcium flocculation by lime softening occurs at pH 11, and flocculates algae by semi-soluble calcium-phosphate hydroxides, reducing algaes charge while bridging cells with its highly charged poly-nuclear ionic species (Ayoub and Koopman, 1986). The targeting of specific anions to precipitate multi-valent cations such as aluminum has also shown promise to cause successful flocculation at higher pH levels. Sulfate and phosphate are stronger negative anions than chlorine ($AlCl_3$) and have better success as coagulant promoters, flocculating negatively charged colloids at higher pH levels (Shin et al., 2008). Aluminum sulfate (Alum) is able to successfully flocculate algae with 150 mg/L at pH 6.5, and calcium phosphate precipitant

successfully flocculated *Scenedesmus dimorphus* at pH 8.5 (Richmond, 1986; Sukenik and Shelef, 1984).

REFERENCES

Adgar A, Cox C, Jones C. 2005. Enhancement of coagulation control using the streaming current detector. Bioprocess and Biosystems Engineering 27(5):349-357.

Aleman J, Chadwick AV, He J, Hess M, Horie K, Jones RG, Kratochvil P, Meisel I, Mita I, Moad G and others. 2007. Definitions of terms relating to the structure and processing of sols, gels, networks, and inorganic-organic hybrid materials (IUPAC Recommendations 2007). Pure and Applied Chemistry 79(10):1801-1827.

Amin S. 2009. Review on biofuel oil and gas production processes from microalgae. Energy Conversion and Management 50(7):1834-1840.

Ayoub GM, Koopman B. 1986. Algal separation by the lime-seawater process. Water Pollution Control Federation 58(9):924-931.

Barsanti L, Gualtieri P. 2006. Algae: Anatomy, biochemistry, and biotechnology. London, UK: Taylor & Francis.

Becker EW. 2008. Microalgae: Biotechnology and microbiology. Cambridge, UK: Cambridge University Press.

Belyea RL, Rausch KD, Tumbleson ME. 2004. Composition of corn and distillers dried grains with solubles from dry grind ethanol processing. Bioresource Technology 94(3):293-298.

Bernhardt H, Clasen J. 1991. Flocculation of micro-organisms. Journal of Water Supply: Research and Technology 40(2):76-87.

Bernhardt H, Lusse B, Hoyer O. 1986. The addition of calcium to reduce the impairment of flocculation by algogenic organic-matter. Journal for Water and Wastewater Research 19(6):219-228.

Bilanovic D, Shelef G, Sukenik A. 1988. Flocculation of microalgae with cationic polymers: Effects of medium salinity. Biomass 17(1):65-76.

Bolto B, Gregory J. 2007. Organic polyelectrolytes in water treatment. Water Research 41(11):2301-2324.

Brown L. 2010. Personal conversation regarding best growth conditions for green microalgae species ed. Texas Agrilife Research. College Station, TX.

Canizares-Villanueva RO, Gonzalez-Moreno S, Dominguez-Bocanegra AR. 2001. Growth, nutrient assimilation and cadmium removal by suspended and immobilized Scenedesmus acutus cultures: Influence of immobilization matrix. Algae and Their Biotechnological Potential:147-161.

Certik M, Shimizu S. 1999. Biosynthesis and regulation of microbial polyunsaturated fatty acid production. Journal of Bioscience and Bioengineering 87(1):1-14.

Chen F, Jiang Y. 2001. Algae and their biotechnological potential: Proceedings of the 4th Asia-Pacific Conference on Algal Biotechnology, 3-6 July 2000 in Hong Kong. Norwell, MA: Kluwer Academic Publishers.

Chen P, Min M, Chen YF, Wang LA, Li YC, Chen Q, Wang CG, Wan YQ, Wang XQ, Cheng YL and others. 2009. Review of the biological and engineering aspects of algae to fuels approach. International Journal of Agricultural and Biological Engineering 2(4):1-30.

Chisti Y. 2007. Biodiesel from microalgae. Biotechnology Advances 25(3):294-306.

Clasen J, Mischke U, Drikas M, Chow C. 2000. An improved method for detecting electrophoretic mobility of algae during the destabilisation process of flocculation: Flocculant demand of different species and the impact of DOC. Journal of Water Supply Research and Technology 49(2):89-101.

Dodd JC. 1979. Algae production and harvesting from animal wastewaters. Agricultural Wastes 1(1):23-37.

Dodd JC, Anderson JL. 1977. Integrated high rate pond algae harvesting system. Progress in Water Technology 9(3):713-726.

Dove A. 2002. Uncorking the biomanufacturing bottleneck. Nature Biotech 20(8):777-779.

Duan J, Gregory J. 2003. Coagulation by hydrolysing metal salts. Advances in Colloid and Interface Science 100-102:475-502.

Dupain X, Costa DJ, Schaverien CJ, Makkee M, Moulijn JA. 2007. Cracking of a rapeseed vegetable oil under realistic FCC conditions. Applied Catalysis B: Environmental 72(1-2):44-61.

Eisenberg DM, Koopman B, Benemann JR, Oswald WJ. 1981. Algal bioflocculation and energy conservation in microalgal sewage ponds. Biotechnology and Bioengineering Symposium 11(1):429–448

Ferell JSR, Valerie R. 2010. National algal biofuels technology roadmap. In: Energy USDOE, editor. Washington, DC: U.S. Dept. of Energy, Office of Energy Efficiency and Renewable Energy, Office of the Biomass Program. p 1-124.

Gouveia L, Oliveira AC. 2009. Microalgae as a raw material for biofuels production. Journal of Industrial Microbiology & Biotechnology 36(2):269-274.

Goyal HB, Seal D, Saxena RC. 2008. Bio-fuels from thermochemical conversion of renewable resources: A review. Renewable and Sustainable Energy Reviews 12(2):504-517.

Graham LE, Wilcox LW. 2000. Algae. Upper Saddle River, NJ: Prentice Hall.

Gregory J, Duan J. 2001. Hydrolyzing metal salts as coagulants. Pure and Applied Chemistry 73(11):1-10.

Gudin CT, C. 1986. Bioconversion of solar energy into organic chemicals by microalgae. Advances in Biotechnological Processes 6:73-110.

Gustafson KR, Cardellina JH, Fuller RW, Weislow OS, Kiser RF, Snader KM, Patterson GML, Boyd MR. 1989. Aids antiviral sulfolipids from cyanobacteria (Blue-Green-Algae). Journal of the National Cancer Institute 81(16):1254-1258.

Hall ES. 1965. The zeta potential of aluminium hydroxide in relation to water treatment coagulation. Journal of Applied Chemistry 15(5):197-205.

Han YM. 2002. Modeling of DAF: the effect of particle and bubble characteristics. London, ROYAUME-UNI: IWA. 8 p.

Harrison RG. 2003. Bioseparations science and engineering. Oxford, UK: Oxford University Press.

Hayden, PL. 1974. Systematic investigation of the hydrolysis and precipitation of aluminum (III). In: Rubin A, editor. Aqueous environmental chemistry of metals. Ann Arbor, MI: Ann Arbor Science Publishers.

Henderson R, Parsons SA, Jefferson B. 2008a. The impact of algal properties and pre-oxidation on solid-liquid separation of algae. Water Research 42(8-9):1827-1845.

Henderson RK, Parsons SA, Jefferson B. 2008b. Successful removal of algae through the control of zeta potential. Separation Science and Technology 43(7):1653 - 1666.

Jensen A. 1984. Excretion of organic carbon as function of nutrient stress: Marine phytoplankton and productivity: Proceedings of the invited lectures to a symposium organized within the 5th Conference of the European Society for Comparative Physiology and Biochemistry - Taormina, Sicily, Italy, September 5-8, 1983. New York, NY: Springer. p 61-72.

Jiang Y, Chen F, Liang S-Z. 1999. Production potential of docosahexaenoic acid by the heterotrophic marine dinoflagellate Crypthecodinium cohnii. Process Biochemistry 34(6-7):633-637.

Kim S, Choi K, Yu P, Kim S, Lee Y. 2008. Evaluation of mixing conditions using an on-line monitoring technique. Korean Journal of Chemical Engineering 25(1):19-24.

Lai RJ, Hudson HE, Singley JE. 1975. Velocity gradient calibration of jar test equipment. Journal American Water Works Association 67(10):553-557.

Lee A, Lewis D, Ashman P. 2009. Microbial flocculation, a potentially low-cost harvesting technique for marine microalgae for the production of biodiesel. Journal of Applied Phycology 21(5):559-567.

Letki AG. 1998. Know when to turn to centrifugal separation. Chemical Engineering Progress 94(9):29-44.

Letterman RD. 1999. Water quality and treatment: A handbook of community water supplies. New York, NY: McGraw-Hill.

McGarry MG. 1970. Algal flocculation with aluminum sulfate and polyelectrolytes. Water Pollution Control Federation 42(5):R191-R201.

McGarry MG, Tongkasame C. 1971. Water reclamation and algae harvesting. Water Pollution Control Federation 43(5):824-835.

Meghzili B, Medjram MS, Zoubida M. 2008. Tests of coagulation - flocculation by aluminium sulphate and polycations Al_{13} on raw waters of the station of treatment Skikda (Algeria). European Journal of Scientific Research: EuroJournals, Inc. p 268-277.

Mohn FH. 1988. Harvesting of micro-algal biomass. In: Borowitzka MA and Borowitzka LJ. editor. Micro-algal biotechnology. Cambridge, MA: Cambridge University Press. p 395 – 414.

Mohn FH. 1980. Experiences and strategies in the recovery of biomass from mass cultures of microalgae. In: Shelef G and Soeder CJ, editor. Algae biomass. Amsterdam: Elsevier/North-Holland Biomedical Press. p 547-571.

Molina G, E., Belarbi EH, Acién Fernández FG, Robles Medina A, Chisti Y. 2003. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnology Advances 20(7-8):491-515.

Moraine R. 1980. Algal mass production as an integral part of a wastewater treatment and reclamation system. In: Shelef G and Soeder CJ, editor. Algae biomass. Amsterdam: Elsevier/North-Holland Biomedical Press.

Morales J, de la Noüe J, Picard G. 1985. Harvesting marine microalgae species by chitosan flocculation. Aquacultural Engineering 4(4):257-270.

MRWA. Coagulation and flocculation coagulation and flocculation process fundamentals: Minnesota Rural Water Association. p 1-6.

Narasimhan. 2010. Microalgal bioremediation of nutrients in wastewater and carbon dioxide in Flue Gas. Rolla, MO: Missouri University of Science and Technology. 78 p.

Nikolov Z. 2010. Personal conversation about algae lipid profiles required for efficient conversion to conventional gasoline. In: Davis R, editor. Personal conversation ed. College Station, TX.

Nobles Jr. DRA, Brown Jr., Malcolm R.; Board of Regents, The University of Texas. 2010. Production and secretion of glucose in photosynthetic prokaryotes (cyanobacteria). US Patent. 7,803,601.

Oh HM, Lee SJ, Park MH, Kim HS, Kim HC, Yoon JH, Kwon GS, Yoon B-D. 2001. Harvesting of Chlorella vulgaris using a bioflocculant from Paenibacillus sp. AM49. Biotechnology Letters 23(15):1229-1234.

Pieterse AJH, Cloot A. 1997. Algal cells and coagulation, flocculation and sedimentation processes. Water Science and Technology 36(4):111-118.

Poelman E, De Pauw N, Jeurissen B. 1997. Potential of electrolytic flocculation for recovery of micro-algae. Resources, Conservation and Recycling 19(1):1-10.

Rasala BA, Muto M, Lee PA, Jager M, Cardoso RMF, Behnke CA, Kirk P, Hokanson CA, Crea R, Mendez M and others. 2010. Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of Chlamydomonas reinhardtii. Plant Biotechnology Journal 8(6):719-733.

Ravina L. 1993. Everything you want to know about coagulation & flocculation. Fourth edition ed. Staunton, VA: Zeta-Meter, Inc.

Richmond A. 1986. CRC Handbook of microalgal mass culture. Boca Raton,FL: CRC Press.

Richmond A. 2004. Handbook of microalgal culture: Biotechnology and applied phycology. Ames, IA: Blackwell Science.

Safar. 1975. Algotherapie. Prackticke Lekarstvi 55:641-648.

Shelef G, Soeder CJ, Balaban M, ule-fituaá¸¥ Mah-lul-máa, Umweltforschung GfrS-u. 1980. Algae biomass: Production and use. Amsterdam: Elsevier/North-Holland Biomedical Press.

Shelef G, Sukenik A, Green M. 1984. Microalgae harvesting and processing: A literature review-A subcontract report: Technion Research and Development Foundation. http://www.nrel.gov/docs/legosti/old/2396.pdf.

Shin JY, Spinette RF, Oâ€™Melia CR. 2008. Stoichiometry of coagulation revisited. Environmental Science & Technology 42(7):2582-2589.

Singh J, Cu S. 2010. Commercialization potential of microalgae for biofuels production. Renewable & Sustainable Energy Reviews 14(9):2596-2610.

Somasundaran P. 2006. Encyclopedia of surface and colloid science. London, UK: Taylor & Francis.

Sukenik A, Shelef G. 1984. Algal autoflocculation—verification and proposed mechanism. Biotechnology and Bioengineering 26(2):142-147.

Svarovsky L. 1990. Solid-liquid separation. Oxford, UK: Butterworths.

Teixeira MR, Rosa MJ. 2006. Comparing dissolved air flotation and conventional sedimentation to remove cyanobacterial cells of Microcystis aeruginosa: Part I: The key operating conditions. Separation and Purification Technology 52(1):84-94.

Tilton RC, Murphy J, Dixon JK. 1972. The flocculation of algae with synthetic polymeric flocculants. Water Research 6(2):155-164.

Uduman N, Qi Y, Danquah MK, Forde GM, Hoadley A. 2010. Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. Journal of Renewable and Sustainable Energy 2(1):012701.

Volesky B. 1990. Biosorption of heavy metals. Boca Raton, FL: CRC Press.

Wakeman RJ, Tarleton ES. 1999. Filtration: Equipment selection, modeling and process simulation. Oxford, UK: Elsevier Advanced Technology.

Widmer MR, McGinnis LM, Stein HH. 2007. Energy, phosphorus, and amino acid digestibility of high protein distillers dried grains and corn germ fed to growing pigs. Journal of Animal Science 85: 2994-3003.

Xiaoying M, Guangming Z, Chang Z, Zisong W, Jian Y, Jianbing L, Guohe H, Hongliang L. 2009. Characteristics of BPA removal from water by PACl-Al13 in coagulation process. Journal of Colloid and Interface Science 337(2):408-413.

APPENDIX 1

Table A.1.1 Composition of Erdschreibers media

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	Pasteurized Sea Water 3L			
2	P-IV Metal Solution	36 mL/3 L		
3	$NaNO_3$ (autoclave before adding) (Fisher BP360-500)	10 mL/3 L	0.7 M	2.3 mM
4	$Na_2HPO_4 \cdot 7H_2O$ (autoclave before adding) (Sigma S-9390)	10 mL/3 L	0.02 M	0.067 mM
6	Vitamin B_{12}	3 mL/3 L		

Metal Solution:

#	Component	Amount	Final Concentration
1	$Na_2EDTA \cdot 2H_2O$ (Sigma ED255)	0.75 g/L	2 mM
2	$FeCl_3 \cdot 6H_2O$ (Sigma 1513)	0.097 g/L	0.36 mM
3	$MnCl_2 \cdot 4H_2O$ (Baker 2540)	0.041 g/L	0.21 mM
4	$ZnCl_2$ (Sigma Z-0152)	0.005 g/L	0.037 mM
5	$CoCl_2 \cdot 6H_2O$ (Sigma C-3169)	0.002 g/L	0.0084 mM
6	$Na_2MoO_4 \cdot 2H_2O$ (J.T. Baker 3764)	0.004 g/L	0.017 mM

Vitamin Solution:

#	Component	Amount
1	HEPES buffer pH 7.8 (Sigma H-3375)	2.4 g/200 mL dH2O
2	Vitamin B_{12} (cyanocobalamin, Sigma V-6629)	0.027 g/200 mL

Table A.1.2. Composition of modified Erdschreibers media:

#	Component	Amount	Final Concentration
1	DI Water 1L		
2	NaCl (Tru Soft, Cas No. 7647-4-5)	15 g/L	0.54 M
3	$NaNO_3$ (Fisher BP360-500)	400 mg	9.2 mM
4	$Na_2HPO_4 \cdot 7H_2O$ (Sigma S-9390)	30 mg	0.2 mM
6	Vitamin Solution	0.5 mL	
7	Metals Solution	1 mL	

Vitamins:
(add 0.5 mL to each 1 L of media) to prepare 1 L Stock Solution

Component	Amount	Volume from stock solutions to Vitamin solution	Final Concentration
thiamine HCl (vit. B_1)	---	200 mg	2.96×10^{-7} M
Component	Amount	Volume from stock solutions to Vitamin solution	Final Concentration
biotin (vit. H)	1.0 g/L dH_2O	1 mL	2.05×10^{-9} M
cyanocobalamin (vit. B_{12})	1.0 g/L dH_2O	1 mL	3.69×10^{-10} M

Trace Metals (add 1ml to each 1 L of media)
To prepare 1 L Stock Solution

Component	Stock solution concentration	Volume added from Stock Solution to Trace Metals Solution	Final Concentration
$FeCl_3$ $6H_2O$	---	3.15 g	1.17 x 10-5 M
Na_2EDTA $2H_2O$	---	4.36 g	1.17 x 10-5 M
$CuSO_4$ $5H_2O$	9.8 g/L dH_2O	1 mL	3.93 x 10-8 M
Na_2MoO_4 $2H_2O$	6.3 g/L dH_2O	1 mL	2.60 x 10-8 M
$ZnSO_4$ $7H_2O$	22.0 g/L dH_2O	1 mL	7.65 x 10-8 M
$CoCl_2$ 6H2O	10.0 g/L dH_2O	1 mL	4.20 x 10-8 M
$MnCl_2$ $4H_2O$	180.0 g/L dH_2O	1 mL	9.10 x 10-7 M

Table A.1.3. Composition of F/2 media

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	$NaNO_3$ (Fisher BP360-500)	**1 mL**	7.5 g/100 mL dH_20	880 µM
2	$NaH_2PO_4 \cdot H_2O$(MCIB 742)	1 mL	0.5 g/100 mL dH_20	36 µM
3	$Na_2SiO_3 \cdot 9H_2O$ (Sigma 307815)	1 mL	3 g/100 mL dH_20	106 µM
#	Component	Amount	Stock Solution Concentration	Final Concentration
4	Trace Metals Solution	1 mL/L		
5	Vitamin Solution	1 mL/L		

Vitamins:
(add 0.5 mL to each 1 L of media) to prepare 1 L Stock Solution

Component	Amount	Volume from stock solutions to Vitamin solution	Final Concentration
thiamine HCl (vit. B_1)	---	200 mg	2.96×10^{-7} M
biotin (vit. H)	1.0 g/L dH_2O	1 mL	2.05×10^{-9} M
cyanocobalamin (vit. B_{12})	1.0 g/L dH_2O	1 mL	3.69×10^{-10} M

Trace Metals (add 1ml to each 1 L of media)
To prepare 1 L Stock Solution

Component	Stock solution concentration	Volume added from Stock Solution to Trace Metals Solution	Final Concentration
$FeCl_3$ $6H_2O$	---	3.15 g	1.17 x 10-5 M
Na_2EDTA $2H_2O$	---	4.36 g	1.17 x 10-5 M
$CuSO_4$ $5H_2O$	9.8 g/L dH2O	1 mL	3.93 x 10-8 M
Na_2MoO_4 $2H_2O$	6.3 g/L dH2O	1 mL	2.60 x 10-8 M
$ZnSO_4$ $7H_2O$	22.0 g/L dH2O	1 mL	7.65 x 10-8 M
$CoCl_2$ $6H_2O$	10.0 g/L dH2O	1 mL	4.20 x 10-8 M
$MnCl_2$ $4H_2O$	180.0 g/L dH2O	1 mL	9.10 x 10-7 M

Table A.1.4. Composition of modified F/2 media

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	$NaNO_3$ (Fisher BP360-500)	**10 mL**	7.5 g/100 mL dH_2O	880 µM
2	$NaH_2PO_4 \cdot H_2O$(MCIB 742)	1 mL	0.5 g/100 mL dH_2O	36 µM
3	$Na_2SiO_3 \cdot 9H_2O$ (Sigma 307815)	1 mL	3 g/100 mL dH_2O	106 µM
4	Trace Metals Solution	1 mL/L		
5	Vitamin Solution	1 mL/L		

Vitamins:
(add 0.5 mL to each 1 L of media) to prepare 1 L Stock Solution

Component	Amount	Volume from stock solutions to Vitamin solution	Final Concentration
thiamine HCl (vit. B_1)	---	200 mg	2.96×10^{-7} M
biotin (vit. H)	1.0 g/L dH_2O	1 mL	2.05×10^{-9} M
cyanocobalamin (vit. B_{12})	1.0 g/L dH_2O	1 mL	3.69×10^{-10} M

Trace Metals (add 1ml to each 1 L of media)
To prepare 1 L Stock Solution

Component	Stock solution concentration	Volume added from Stock Solution to Trace Metals Solution	Final Concentration
$FeCl_3$ $6H_2O$	---	3.15 g	1.17 x 10-5 M
Na_2EDTA $2H_2O$	---	4.36 g	1.17 x 10-5 M
$CuSO_4$ $5H_2O$	9.8 g/L dH_2O	1 mL	3.93 x 10-8 M
Na_2MoO_4 $2H_2O$	6.3 g/L dH_2O	1 mL	2.60 x 10-8 M
$ZnSO_4$ $7H_2O$	22.0 g/L dH_2O	1 mL	7.65 x 10-8 M
$CoCl_2$ $6H_2O$	10.0 g/L dH_2O	1 mL	4.20 x 10-8 M
$MnCl_2$ $4H_2O$	180.0 g/L dH_2O	1 mL	9.10 x 10-7 M

APPENDIX 2

Optical Density (OD) measurement SOP

1.0 Equipment

1.1 Ultraviolet-visible (uv-vis) spectrophotometer

1.2 Cuvette, 3 cm x 1 cm

1.3 Calibrated pipette

2.0 Calibration

2.1 Calibration and verification performed biannually by Varian

3.0 Reagents

3.1 De-Ionized (DI) H_2O

4.0 Procedure

4.1 Dilute culture sample with DI H_2O to desired concentration that falls within the linear range on the spectrophotometer for the specific culture.

4.2 Run DI H_2O as blank to zero spectrophotometer

4.3 After blank is complete, gently mix diluted culture sample in cuvette and run immediately.

4.4 Use the absorbance value at 750 nm.

Hemocytometer SOP

1. Equipment

1.1 Inverted microscope with 10X objective

1.2 Pipetman, 1-20 µl

1.3 Hemocytometer with cover glass

1.4 Prepare clean, dry hemocytometer with the cover slip.

2. Method:

2.1 Dilute a sample of cells 1: 10 with DI water and transfer 10 µl (0.01 ml) of the diluted cell suspension into the hemocytometers chamber

2.2 Using the 10X objective, focus on the gridlines of the chamber. Count the viable cells in three random squares of one chamber. Cells that lie on the lines should only be counted if they are toughing the top and left-hand lines of each corner square.

3. Clean the hemocytometer by rinsing with dH_2O then 70% IPA and drying thoroughly with a kim-wipe.

4. Equations:

3.1 Cells/ml = Total cells counted in 3 squares X 3,333 X [any dilution made]

DW/AFDW SOP

1.0 Pre Ashing Filters and Trays

Filters and Trays are ashed to remove all moisture and impurities that would be lost in subsequent drying and ashing steps.

1.1 Make an envelope for 3 filters with aluminum foil using latex gloves. Make sure to fold all edges of envelopes.

1.1.1 Always Use forceps to handle filters and trays

1.2 Turn three aluminum weigh trays over and indent an identification mark with a blunt rubber object.

1.3 Place the three labeled trays on a stainless steel tray that has been wrapped in aluminum foil. Cover the aluminum trays with aluminum foil.

1.4 Turn on muffle furnace to 500 °C

1.5 Once muffle furnace has reached 500 °C place covered trays and enveloped filters in muffle furnace for one hour.

1.6 After one hour of incubation turn off muffle furnace and wait for it to cool down. Turn on muffle furnace periodically to check its temperature on the digital meter in the furnace until the unit cools to 300 °C.

1.7 Carefully remove the trays and filters from the furnace with hot mill gloves and a large pair on forceps or tongs. Place the trays on a cool metal table to quickly cool until the aluminum foil can be removed and the trays can be moved from the tray to the vacuum desiccator with hot mill gloves and forceps.

1.7.1 It is important to never touch the individual metal trays or filters with the hot mill gloves, and to limit handling with latex gloves. It is best to only handle the trays and filters with forceps washed in IPA.

1.8 When weighing aluminum dishes and filters that have been dried by the blast furnace (500 °C) or drying oven (105 °C) wait till the filter weight stabilizes for at least 5 seconds before recording weight. Weigh dishes and especially

filters will accumulate water from ambient air while sitting on the scale. It is has also been found useful to use a vacuum desiccator.

2.0 Weighing pre-ashed filters and trays

2.1 Once the trays and filters have cooled to room temperature inside the desiccator, record one of the trays weight with an analytical balance as weight 1 (WT1).

2.1.1 Wait until the filter and or tray weight stabilizes for at least 5 seconds before recording the weight. The filter will accumulate water from ambient air while sitting on the scale. This method of weighing should be done for all subsequent weighting's.

2.2 Add a filter to the tray already on the analytical balance and weigh the tray and filter together as weight 2 (WT2).

2.2.1 Once done weighing the tray and filter immediately place back under the covering aluminum foil and place in back the desiccator as soon as possible.

2.2.2 Rinse forceps with 70% IPA before and after every time you handle the filters.

2.3 Repeat the weighing for all trays and filters.

3.0 Filtering samples

3.1 Assemble glass filter assembly.

3.2 Using forceps cleaned with 70% IPA seat a filter on the filter assembly with the filters rough side facing up. The rough side, which will be exposed to the filtering algae, is more efficient at fixing algae to the filter than the smooth end.

3.3 Seat the other 2 filters as well and turn on the vacuum with the filter valves closed

3.4 Wet the filters with 5 mL of 0.5M ammonium formate.

3.5 Open the filter valve and immediately filter 40mL of algae.

3.5.1 Always filter 40 mL of algae despite what the proposed dry weight is unless it is at or above 3 grams/L. The more biomass fixed to the filter will give better weighing results.

3.5.2 Samples should be sampled within 30 minutes of collecting.

3.6 Before the filter completely dries, wash the sides of the filter funnels with 10 mL of 0.5M ammonium formate.

3.7 When the filter is dry shutoff the filter vacuum valve, then transfer filter into the correct aluminum weigh dish and cover with aluminum foil.

4.0 Drying filters for dry weight measurement

4.1 Turn on drying oven to 115 ^{o}C

4.2 Place aluminum trays and filters in drying oven heated to 115 ^{o}C.

4.2.1 Make sure that filters are uncovered

4.3 Remove trays with filters and place back into desiccator to allow them to cool to room temperature.

4.4 Once trays and filters have cooled to room temperature remove the tray and filter from the desiccator with washed forceps and weigh the tray and filter together as weight 3 (WT3).

4.5 Repeat for other tray and filters.

4.6 Keep all trays and filters covered when not weighing and place all samples back into the desiccator as soon as possible.

5.0 Ashing trays and filters for ash free dry weight

5.1 Turn on the muffle furnace to 500 ^{o}C

5.2 Once muffle furnace has reached 500 ^{o}C remove trays and filters from desiccator and place on a stainless steel tray and completely cover the stainless steel tray with aluminum foil and place into the muffle furnace with a pair of hot mill gloves and a large pair of forceps or tongs in a quick manner. Keep the muffle furnace with the tray inside on for one hour.

5.3 After one hour of incubation turn off muffle furnace and wait for it to cool down. Turn on muffle furnace periodically to check its temperature on the digital meter in the furnace until the unit cools to 300 ^{o}C.

5.4 Remove the tray with a pair of hot mill gloves and a large pair of forceps or tongs in a quick manner. Place the trays on a cool metal table to quickly cool until the aluminum foil can be removed and the trays can be moved from the

tray to the vacuum desiccator with hot mill gloves and forceps. Allow the aluminum trays and filters to cool to room temperature.

5.5 Once trays and filters have cooled to room temperature remove the tray and filter from the desiccator with washed forceps and weigh the tray and filter together as weight 4 (WT4).

5.6 Repeat for other tray and filters.

5.7 Keep all trays and filters covered when not weighing and place all samples back into the desiccator as soon as possible.

5.8 When weighing aluminum dishes and filters that have been dried by the blast furnace (500 °C) or drying oven (105 °C) wait till the filter weight stabilizes for at least 5 seconds before recording weight. Weigh dishes and especially filters will accumulate water from ambient air while sitting on the scale.

6.0 Calculations

6.1 Dry Weight (DW) = (WT3 – WT2)/(mL filtered)*1000

6.2 Ash Free Dry Weight (AFDW) = (WT 4 – WT3)/(mL filtered)*1000

APPENDIX 3

Zeta Potential measurement method development

The purpose of this data and discussion is to determine the effects that altering the samples density and dilution method had on the samples measured zeta potential. It is important to determine all of the effects that sample preparation can have on zeta potential measurements in order to validate an experimental method.

It is important to control all variables that can affect zeta potential measurements while manipulating the sample pH and flocculant dosages. Zeta potential measurements are only relevant to the method used and can only be repeated if using the same method of measurement. Several other methods use different buffers and densities. Further research was done to better understand how some of the variables involved with sample preparation may change a samples zeta potential from its natural state and what, if any sample preparation was necessary.

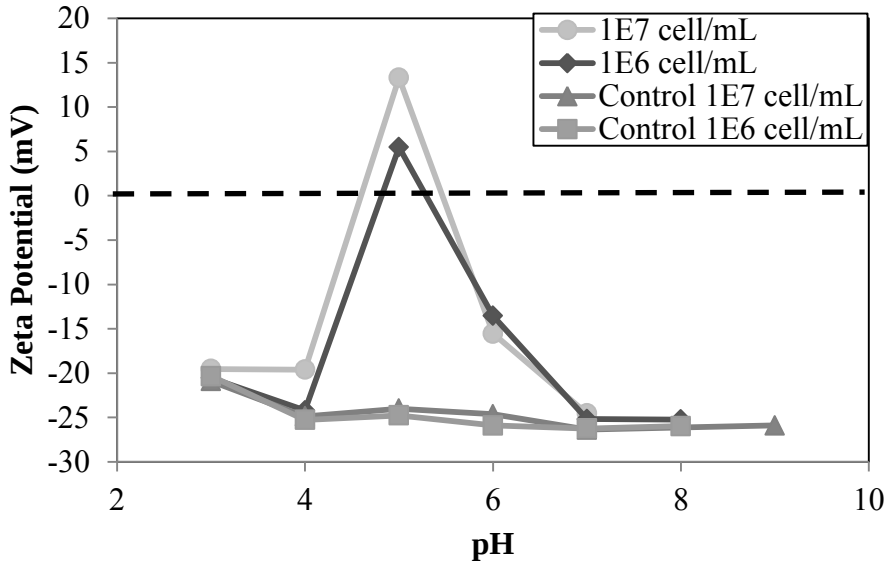


Figure A.3.1. Effect of dilution on Zeta potential with 0.5 g/L $AlCl_3$ and 10^6 cell/mL.

Figure A.3.1. shows the charge neutralization of two different dilutions of *N.oculata* with 0.5 g/L $AlCl_3$. As seen in Figure A.3.1 the two conentrations of cells at 1E7 and $1*10^6$ cells/mL show similar zeta potential values at all pH points except for pH 4, where the difference is possibly due to zeta potential measurement error with a difference of -4.6 mv, and at pH 5 whose difference is conceringly larger difference -7.83 mV. This indicates the ability to dilute algaes natural environment without altering the measured zeta potential as long as NaCl concentration is noted as discussed above. Changing the algaes environment by dilution is not advantageous though as it changes the algaes natural environment, and introduces more room for error when preparing samples for zeta potential measurement.

Samples were prepared by first diluting the culture before adding the $AlCl_3$ flocculant as described in the material and methods section. The process of diluting to

$1*10^6$ cells/mL inhibits the ability to measure algae settling rate and removal efficiency during harvesting by flocculation, as the low OD values give bad measurement resolution. An alternative is to dilute samples according to the post dosing dilution method for zeta potential measurement as described in the materials and methods. Samples would be taken from a 1E7 cells/mL culture, which gives good settling rate and removal efficiency data by flocculation, and then diluted to $1*10^6$ cells/mL with the standardized media. Dilution can give better zeta potential data if conductivity is reduced which interferes with zeta potential measurement as described in the discussion section. It was decided to dilute with the original media in an effort to conserve the cultures high conductivity.

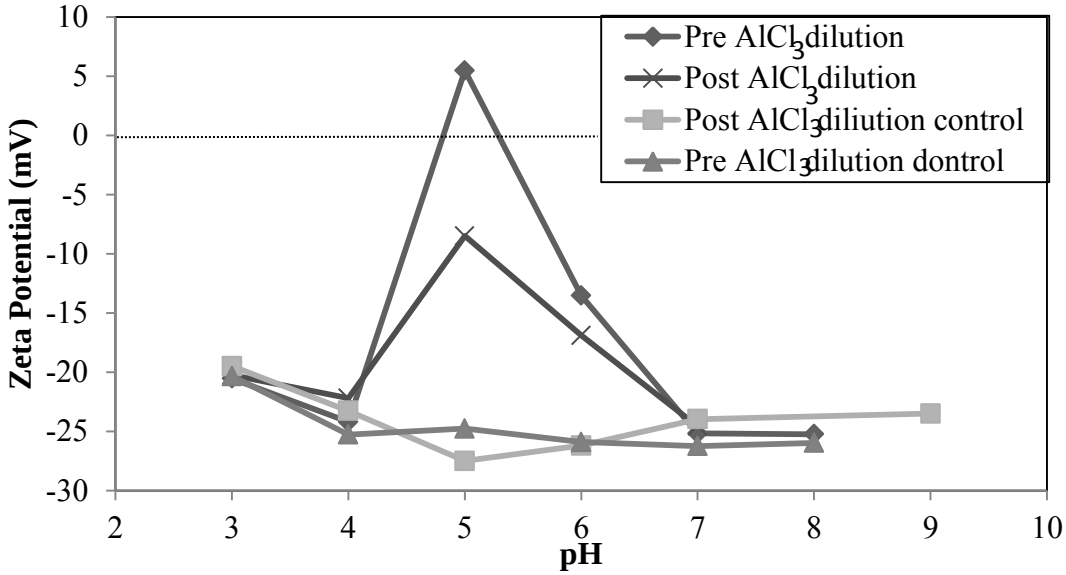


Figure A.3.2. Effect of type of dilution on zeta potential with 0.5 g/L $AlCl_3$.

Figure A.3.2 displays the effect of diluting before and after $AlCl_3$ dosing on zeta potential measurement. As seen in Figure A.3.1, zeta potential values all appear similar except at pH 5, where the difference in charge neutraliztion is large, especially at pH 5. At pH 5 with 0.5 g/L $AlCl_3$, the sample with dilution after $AlCl_3$ addition shows a zeta potential of -8.46 mV where as the sample with dilution before $AlCl_3$ addition, measures a zeta potential of 5.47 mV, a difference of 13.96 mV. This difference indicates that any dilution of samples for zeta potential measurement should be done before $AlCl_3$ to keep values similar to the dense non altered algae samples as shown in Figure 2.7.

APPENDIX 4

Figure A.4.1. PB-900, Phipps and Bird 6 paddle programmable jar tester with 2 liter jars with sampling ports (Courtesy of Phipps & Bird inc.).

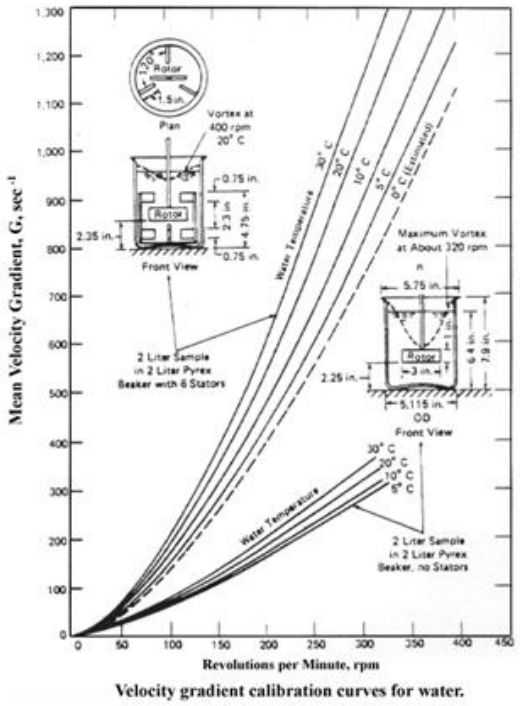


Figure A.4.2. Velocity gradient G (1/s) curves for determining shear imparted on flocks vs. paddle speed (rpm) (Courtesy of Phipps & Bird, Inc.).

Figure A.4.3. Folded capillary electrophoresis cell with gold plated electrodes (Courtesy of Malvern Instruments Ltd).

Figure A.4.4. Malvern Zetasizer laser diffraction electrophoresis instrument. (Courtesy of Malvern Instruments Ltd).

APPENDIX 5

A.5.1 Ultrasonic concentration

An advance form of algae harvesting and concentration by coagulation employs the use of a low energy ultrasonic standing waves to force cells together at the lower pressure areas of the nodes of the waves. The cells can aggregate into large flocks capable of settling quickly when forced together by the ultrasonic standing waves. The technique does not foul the biomass with polymers or metallic ions, does not shear cells unless tailored to, and can in theory be used in tandem with ionic flocculation to increase efficiency or decrease the required energy to cause flocculation with counter ion charge neutralization. The process can also be tailored to shear cells once concentrated at the nodes of the ultrasonic waves by specifically altering the frequency of the waves. Unfortunately the technology is still being developed and requires high power consumption while yielding a low concentration factor compared to centrifugation and other concentration processes (Chen et al., 2009).

A.5.2 Dissolved Air Flotation (DAF) Harvesting

The visual confirmation of flocks settling out of suspension is not enough to justify the use of flocculation as an initial harvesting step. Flocculation must have a high removal efficiency, concentration factor, and settling rate. The force impacted upon flocks and destabilized colloids by gravity at 9.8 m/s^2 is sometimes not enough to give sufficient removal efficiencies, concentration factors, or settling rates. Centrifugation is

commonly used with flocculated aggregates to increase the gravitational force on flocks for quicker and higher removal efficiency. One method to increase these standards is to use removal techniques other than simple gravity settling such as dissolved air flotation (DAF), electrolytic flocculation, and ultrasonic concentration. Each method is able to impart forces other than gravity on algae flocks to increase flocculation.

DAF is commonly used in waste water treatment processes and gives increased algae removal efficiency with lower flocculant dosage over sedimentation (Henderson et al., 2008b). DAF works by injecting air saturated culture at the bottom of a culture allowing micro bubbles from the injected saturated culture to float upwards, binding to algae aggregates and concentrating them at the top of the culture in an algal froth which can be removed by skimming, as displayed in Figure 2.10 (Han et al., 2002). DAF is commonly combined with coagulation where cells will become chemically encouraged to form aggregates that can be easily removed by force but will not settle rapidly under gravity (Chen et al., 2009). Algae aggregates can float to the surface more efficiently than they would normally settle under gravity making DAF quicker than gravity settling (Teixeira and Rosa, 2006).

The absence of water imparting a downward force on settled algae allows for a higher solids fraction in the concentrated algae of DAF with harvested concentrations up to 7% (Becker, 2008). This is considerably higher than solid fractions from settling which yield 1.5% to 3% TSS (Eisenberg et al., 1981; Mohn., 1980). To make the smallest bubbles possible, which have the best removal efficiency, bubbles can be formed by effervescence from a supersaturated solution or by electrolysis which causes

small oxygen and hydrogen bubbles (Uduman et al., 2010). Small bench scale DAF jar testers are used to optimize the flocculation process by determining key parameters for successful flocculation such as air pressure, flow rate, flocculant dosage, and recycling ratios for scale up (Henderson et al., 2008b). The cost of producing effective micro bubbles and skimming devices necessitates economic analysis before implementation of DAF concentration processes. It is also advantageous to fully understand the science behind flocculation and settling before relying on DAF's higher removal efficiency (Henderson et al., 2008b).

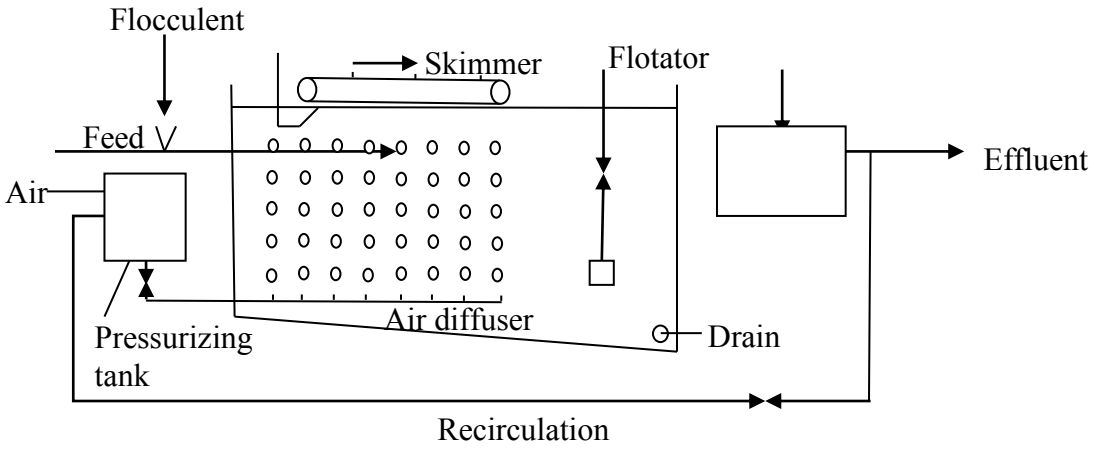


Figure A.5.1. Diagram of dissolved air flotation harvester (Becker, 2008).

VITA

Ryan T. Davis
c/o Dr. Zivko Nikolov
MS 2117 TAMU
College Station, Texas 77843-2117

Email Address: AlgFuel@gmail.com
Phone: 951-Alg-Fuel

Education

Master of Science in Biological and Agricultural Engineering
Texas A&M University (August 2011)
Bachelor of Science in Biological Engineering
University of Arizona (May 2008)

Certifications

Texas E.I.T. (Environmental Engineering)
Business management certificate, Mays Business School, Texas A&M University

List of Professional Presentations

Algal Biomass Organization 4th Annual Biomass Summit
Harvesting Algae by Ionic Coagulation, Phoenix, AZ, 2010

International Conference on Algal Biomass, Biofuels and Bioproducts
Harvesting of N. oculata and N. salina by electrolyte flocculation,
St. Louis, MO, 2011